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An adapted *in vitro* assay to assess *Campylobacter jejuni* interaction with intestinal epithelial cells: Taking into stimulation with TNFα

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ABSTRACT

Campylobacter jejuni is the most prevalent foodborne bacterial infection agent. This pathogen seems also involved in inflammatory bowel diseases in which pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), play a major role. *C. jejuni* pathogenicity has been extensively studied using *in vitro* cell culture methods, and more precisely "healthy" cells. In fact, no information is available regarding the behavior of *C. jejuni* in contact with TNFα-stimulated cells. Therefore, this research was designed to investigate the effect of TNFα on *C. jejuni* interaction with human intestinal epithelial cells (HT29 and HT29-MTX). To ensure IL-8 production induced by TNFα, human rtTNFα was added to HT29 and HT29-MTX before adhesion and invasion assays. About 10^8 CFU bacteria of *C. jejuni* strains cells were added to measure their adherence and invasion abilities using TNFα-stimulated cells *versus* non stimulated cells. Exposure to TNFα results in IL-8 overproduction by intestinal epithelial cells. In addition, the effect of TNFα pre-treatment on *C. jejuni* adhesion and internalization into eukaryotic cells is strain-dependent. Indeed, the adhesion/invasion process is affected in < 50% of the strains tested when TNFα is added to the intestinal cells. Interestingly, TNFα affects more strains in their ability to adhere to and invade the mucus-secreting HT29-MTX cells. Among the 10 strains tested, the aero-tolerant *C. jejuni* Bf strain is one of the most virulent. These results suggest that the TNFα signalling pathway could participate in the internalization of *C. jejuni* in human intestinal cells and can help in understanding the pathogenicity of this microorganism in contact with TNFα-stimulated cells.

1. Introduction

Campylobacter jejuni is the most common cause of bacterial foodborne infections in developed countries (Epps et al., 2013; Golz et al., 2014), leading to campylobacteriosis, the most frequently reported zoonosis in the European Union (EU) (EFSA, 2017). Campylobacteriosis is generally characterized by diarrhea, abdominal pain, and fever. In rare cases, this infection can lead to the development of more serious complications such as reactive arthritis and Guillain–Barré and Miller–Fisher syndromes (Fica et al., 2011; Kuwabara, 2011). Once ingested by the host, *C. jejuni* pathogenesis involves several steps (Young et al., 2007; Rodrigues et al., 2015a): from adhesion to cell surface, followed by internalization within the intestinal host cells, to survival into vacuoles and translocation (Hu and Kopecko, 2000). Due to the absence of a simple and reliable animal model that mimics human *Campylobacter* spp. infections, *in vitro* cell culture methods, such as epithelial intestinal cells, have been used more extensively to study the pathogenicity of *C. jejuni* (Janssen et al., 2008; Rodrigues et al., 2015a). Studies using human intestinal epithelial cells like HT 29, CaCo 2 and T84, resulted in a better understanding of *Campylobacter* pathogenesis and showed that invasion of intestinal cells is a very important step (MacCallum et al., 2006, John et al., 2017). Invasion and adhesion tests with HT 29 cells allow to measure adhesion and invasion abilities of *Campylobacter* strains using a gentamycin protection assay, by calculating index numbers after plating adhesive and invasive bacteria. (Rodrigues et al., 2015a).

Adhesion to and invasion of intestinal cells by *C. jejuni* induce secretion of interleukin 8 (IL-8), IL-1, IL-6, interferon-gamma (IFN-γ), transforming growth factor (TGF-β), tumor necrosis factor alpha (TNFα), IL-4, and IL-10, which enables the recruitment of neutrophils and macrophages (Hickey et al., 2000; Al-Salloom et al., 2003; MacCallum et al., 2006; Al-Amri et al., 2008). These host-inflammatory responses initiated by intestinal epithelial cells are likely to contribute significantly to the pathology observed (MacCallum et al., 2006). *C. jejuni* is also suspected of being involved in the initiation and/or exacerbation of inflammatory bowel diseases (IBD), including Crohn's

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disease and ulcerative colitis, and irritable bowel syndrome (IBS) (Mylonaki et al., 2004; García Rodriguez et al., 2006; Gradel et al., 2009; Kalischuk and Buret, 2010). Several studies suggested that *C. jejuni* infection may be associated with increased IBD risk and flare-ups of IBD (Gradel et al., 2009; Jess et al., 2010). Chronic intestinal inflammation enhances TNFα levels in the host (Andoh et al., 2008), leading to a potential exposition of *C. jejuni* to TNFα. Stimulation of intestinal epithelial cells with TNFα has been extensively used to induce a set of epithelial cell responses that are related to initiating and sustaining mucosal inflammation. This stimulation causes the increased expression and secretion of a number of cytokines with chemoattractant and pro-inflammatory functions (Kagnoff and Eckmann, 1997); of these, IL-8 was suggested as the initial signal for the acute inflammatory response that follows bacterial invasion of mucosal surfaces (Eckmann et al., 1995; Eckmann and Kagnoff, 2005). TNFα can affect bacterial invasion into eukaryotic cells by various mechanisms, such as complexation with the bacteria, presence of specific bacterial cell-wall components, a more efficient signal transduction mechanism, or a modification of bacterial gene expression, and this is linked to the inflammatory state of the cells (Ma et al., 2010). For instance, *Salmonella enterica* Typhimurium pre-treated with TNFα induced more bacteria internalization and a more severe inflammatory response in intestinal epithelial cells than untreated pathogen (Ma et al., 2010). In addition, in *Salmonella* Dublin, *S.* Typhimurium, and enteroinvasive *Escherichia coli*, the TNFα facilitates internalization into intestinal epithelial cells and destruction of bacteria through oxidative and nitrosative stresses (Kim et al., 2010), and initiation of the inflammatory response.

Despite the large number of studies that have employed *in vitro* cell models to investigate separately *C. jejuni* virulence or pro-inflammatory cytokine induction, no information is available regarding the virulence of *C. jejuni* after contact with inflamed cells after stimulation by TNFα. In the current study, we examined the impact of TNFα on the level of *C. jejuni* internalization by intestinal epithelial cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains used in this study are presented in Table 1. Before each experiment, the strains were grown under microaerobic conditions (10% carbon dioxide (CO_2), 5% oxygen (O_2), and 85% nitrogen (N_2) at 42 °C on Karmali agar plates (Oxoid, Dardilly, France) for 24 h (h). The microaerobic gaseous atmosphere was obtained using gas replacement jars (GRJ) 4 X flushed/filled with a MACS MICS system (Whitley jar gassing system) at −50 kPa vacuum.

2.2. Culture of eukaryotic cell lines

Two intestinal cell lines were used: HT29 cells, derived from a cell line originating from colorectal carcinoma isolated in 1964 (Naughton et al., 2013), and HT29-MTX cells originating from an HT29 subclone, which were differentiated into mature caliciform cells using methotrexate (MTX) (Lesuffleur et al., 1993).

Cells were maintained and assays were conducted according to Haddad et al. (2010a, b). Briefly, the eukaryotic cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), containing 200 mM L-glutamine, 250 μg.mL^{-1} gentamicin (Sigma Aldrich, Saint-Louis, USA) and 2.5 μg mL^{-1} amphotericin B (Sigma Aldrich, Saint-Louis, USA). The cells were grown routinely in tissue culture flasks at 37 °C in a 5% CO_2-humidified atmosphere. Cells were passaged when the confluence of the flasks was about 80–100%. Trypsin treatments were carried out using 0.25% trypsin-phosphate buffered-saline solution (PBS, Eurobio, Courtaboeuf, France) at 37 °C in a 5% CO_2-humidified atmosphere.

2.3. Control of HT29 cell inflammation

Estimation of the amount of IL-8 produced by epithelial cells was used as a marker of the inflammation status of HT29 cells, induced by TNFα. Böcker et al. (2000) have already demonstrated that IL-8 secretion was stimulated by 10 ng mL^{-1} of TNFα in both HT29 and HT29-MTX, with no marked difference in the response between the two cell lines. To induce IL-8 secretion, 10 ng mL^{-1} of human rtTNFα (Promega, Madison, USA) was added to the eukaryotic cells before adhesion and invasion assays. This step is called “pre-treatment” throughout the article. The level of IL-8 production by TNFα stimulation was quantified using the ELISA MAXTM Standard Sets Human IL-8 kit (BioLegend) every hour for 4 h according to the supplier's instructions.

We tested whether removing TNFα from the culture medium enabled the IL-8 level to be maintained. For this, after 4 h of pre-treatment of the cells with TNFα, cells were washed to eliminate TNFα from the culture medium. Then IL-8 concentration was measured one and two hours post-washing as described previously.

2.4. Infection of HT29 cells: adhesion and invasion assays

In vitro virulence assays were conducted according to Haddad et al. (2010a, b) with some modifications. Briefly, each well of a 24-well tissue culture tray was seeded with approximately 10^5 eukaryotic cells and incubated for 5 days at 37 °C in a 5% CO_2 humidified atmosphere. After washing, cells were infected with a suspension of approximately 10^8 CFU bacteria (*multiplicity of infection MOI = 1000*). To assess the adhesion step, the infected monolayers were incubated for 1 h at 37 °C in a humidified 5% CO_2 incubator. The cell monolayer was rinsed, and lysed by addition of 0.5 mL Triton X100 0.1% (Labo-Si, Paris, France) at room temperature for 30 min. For the invasion assays, infected-tissue culture plates were incubated at 37 °C in 5% CO_2 for 3 h, gentamicin (250 μg ml^{-1}) (Sigma, Saint Quentin Fallavier, France) was added to the wells for 1 h 45 min to kill extracellular *C. jejuni*. The infected monolayers were rinsed lysed by addition of Triton ×100 0.1% at room temperature for 30 min.

For adhesion and invasion assays using TNFα-stimulated cells, the eukaryotic cells were pre-treated for 4 h with 10 ng mL^{-1} of TNFα. After this pre-treatment, cells were infected with a suspension of approximately 10^8 CFU bacteria. For information, TNFα was added to each well after all washing steps, to keep the concentration of the reagent during all stages of the experiment to ensure IL-8 production.

C. jejuni enumeration was performed on Karmali agar plates using the microdroplet technique (Morton, 2001) followed by incubation at 42 °C for 48 h under microaerobic conditions. The percentage of adhesion and invasion was calculated by dividing the number of adhered or invaded bacteria by the initial inoculum of bacteria. The results obtained in adhesion and invasion assays using TNFα-stimulated cells were compared to controls (adhesion and invasion assays on non TNF stimulated HT29 and HT29-MTX cells).

Table 1
Strains used in this study.

C. jejuni strain	Origin	Reference
Bf	Human	Rodrigues et al., 2015b; Bronnec et al., 2016a, b
Subsp. *jejuni* NCTC 11168 = ATCC 700819	Human	Parkhill et al., 2000
RM1221	Animal	Fouts et al., 2005
Subsp. *jejuni* 81–176	Human	Hofreuter et al., 2006
Subsp. *doylei* 269.97, RM4099	Animal	Fouts et al., 2007
Subsp. *jejuni* 81,116; NCTC 11828	Human	Pearson et al., 2007
Subsp. *jejuni* 00–2538	Human	Clark et al., 2014
Subsp. *jejuni* 00–2544	Human	Clark et al., 2014
Subsp. *jejuni* 00–2426	Human	Clark et al., 2014
Subsp. *jejuni* 00–2425	Human	Clark et al., 2014