Participation of the Cytoskeletal and Lysosomal Compartments in *Campylobacter jejuni* Invasion of Caco-2 cells, the Cellular Response by Morphometric Analysis and the Presence of Cytokine and Chemokine Transcripts

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Abstract This study aimed to evaluate the participation of actin and tubulin in the process of internalisation, the interaction of bacterial phagosomes with lysosomes, the morphometric changes and the expression of inflammatory cytokines in Caco-2 cells infected with *Campylobacter jejuni*. Both actin and tubulin participated in the process of internalisation. Inside the cells, lysosomes fuse with phagosomes, which may lead to bacterial death because after 2 h, the bacteria were not detected by Transmission electron microscopy (TEM). There is increased expression of TGF- β 3 during the early stages, and IL-8 was expressed after 60 min p.i. This work showed that *C. jejuni* invades and causes major morphometric changes in epithelial cells. In response, the cells increase their expression of cytokines that can lead to inflammation. The mechanisms of invasion are dependent on actin and tubulin, and once internalised, lysosomes fuse with phagosomes.

Keywords IL-8 · TGF- β 3 · Lysosomes · Cytoskeleton · Invasion

Introduction

In recent years, advances have been made in increasing our understanding of the interactions between *C. jejuni* and host cells. The participation of the cytoskeleton in the invasion process is still under discussion. Another process not yet fully understood is the survival of bacteria within epithelial cells; the route of intracellular *C. jejuni* in epithelial cells is very different.

In response to bacterial infection, eukaryotic cells are distended due to the presence of a cytotoxin designated as the “cytolethal distending toxin” (CDT) [21, 28]. The induction of inflammatory and pro-inflammatory cytokines is important for the successful defence against pathogens in the body [7, 15].

It is important to understand *C. jejuni* invasion and the host cell response in order to prevent and control bacteria in humans because of the high incidence of and the occurrence of severe autoimmune diseases after infection. The aims of this study were to evaluate the process and route of invasion, the fusion of lysosomes with bacterial vacuoles, the morphological changes in cells in the presence of bacteria and the induction of increased transcription levels of inflammatory cytokines by *C. jejuni* in Caco-2 cells.

Experimental Procedures

Bacteria

For this study, the *C. jejuni* subsp. *jejuni* (IAL 2383) isolated from a human outbreak and deposited in the Banco de

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Culturas do Instituto Adolfo Lutz, São Paulo (IAL, Brazil) was used. This strain shows the main virulence genes of *C. jejuni* (data not shown). The bacteria were diluted in Bolton broth (Oxoid) under microaerobic conditions (Probac microaerobic generator) at 37 °C for 24 h. Subsequent cultivation was performed on solid mCCDA medium (Oxoid) under microaerobic conditions (Probac microaerobic generator) at 37 °C for 42 h.

Caco-2 Cells

We used polarised Caco-2 cells (human colon adenocarcinoma) in culture in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10 % foetal bovine serum and incubated at 37 °C with 5 % CO₂ for 10 days before experimentation.

Internalisation of *C. jejuni* in Caco-2 cells

Polarised Caco-2 cells were grown in bottles and infected with 2×10^7 CFU from *C. jejuni* diluted in saline solution and analysed after 5, 15, 30 and 60 min (min), 2 and 4 h. The experiment was performed in triplicate with two replications. At all time points, a control group was inoculated with sterile saline without bacteria. For each period, the cells were removed with a cell scraper and washed by centrifugation ($200 \times g$) for 5 min.

To remove the bacteria that were attached but not internalised, the fragments were washed 5 times with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium ($200 \times g$) for 5 min. The cells were treated with gentamicin (100 mg/ml) for 60 min to kill the attached bacteria, washed 5 times with HBSS for 10 min, and centrifuged at $13,500 \times g$ to remove the bacterial DNA that was not internalised. After this period, the cells were treated with 1 ml 1 % triton x-100 for 30 min to release the internalised bacteria. The samples were diluted at a ratio of 1:10 and analysed with real-time PCR to quantify the internalised bacteria.

Quantification of *C. jejuni* Using Real-Time PCR

The real-time PCR assay was performed using the BAX System (Dupont, Wilmington) according to the manufacturer's procedures [30]. The tubes were transferred to the thermocycler and run through the pre-established program. At the end of the amplification and detection cycles, the equipment issued the results identifying the species and number as CFU/g. The ATCC 33291 strain of *C. jejuni* was processed in parallel as a positive control and water was used as negative control.

Participation of the Cytoskeleton in the Invasion of *C. jejuni*

To investigate the involvement of actin filaments in the process of internalisation, we incubated polarised Caco-2 cells in 5 µM cytochalasin D: In Group 1 (positive control) consisted of cells not treated with cytochalasin but infected with *C. jejuni*. Group 2 consisted of Caco-2 cells treated with cytochalasin and infected with *C. jejuni*. Group 3 consisted of cells treated with cytochalasin but not infected with *C. jejuni*. After the 60 min cytochalasin treatment, the drug was withdrawn in groups 2 and 3, and the cells were washed with PBS. Next, the means of three groups were exchanged, and groups 1 and 2 were infected with 2×10^7 CFU of *C. jejuni* per bottle and evaluated by real-time PCR at 5, 15, 30 and 60 min and 2 and 4 h post infection (p.i.).

Similar experiments were conducted to evaluate the participation of microtubules using nocodazole (10 µM), and the groups were evaluated at 30 min and 4 h p.i.

Campylobacter jejuni was available using the same protocol used to assess the internalisation as described above: "Internalisation of *C. jejuni* in Caco-2 cells". The experiment was performed in triplicate with two replications

Morphometric Analysis of Caco-2 Cells Infected with *C. jejuni*

To examine morphological changes, the Caco-2 cells were seeded in 24-well plates at a density of 3.5×10^5 cells/ml, incubated for 7 and after. After period, Caco-2 were infected in triplicate with 2×10^7 CFU per well; there was a negative group in parallel. At 30 and 60 min. p.i., the cells were stained with Giemsa for 40 min. All coverslips were analysed by computer analysis of digitised images obtained from an Olympus BX 40 microscope with a 100× objective coupled to an Olympus camera (Oly 200) and connected to a PC via the PC card scanner Datatranslation 3153. From each coverslip using the software Image HL (Western Vision Software), approximately 36 epithelial cells from 6 to 8 random and different fields of each cell were measured in addition to the longest perpendicular axis.

Invasion Rate and Assessment of Fusion of Lysosomal Compartments with Vacuoles Containing *C. jejuni*

Polarised Caco-2 monolayers were prepared by seeding 10^5 cells on a Transwell clear polyester membrane (pore size, 3 µm; diameter, 6.5 mm; Millipore Corp.), followed by growth for 10 days. The tests were performed in duplicate for the test group, and a negative control was used. The cells from the test group were infected with 2×10^7 CFU *C. jejuni* per well.

At 15, 30 and 60 min. and 2 and 4 h p.i., the membranes containing the cells were removed, washed and fixed in a 1 % Karnovsk solution for 60 min. For labelling of lysosomal compartments, we used acid phosphatase according to the protocol of Worth et al. (2009).

The material was dehydrated in alcohol, and the samples were subsequently processed in LR White. Ultrathin sections were contrasted by uranyl acetate and lead nitrate on nickel small screens. The small screens were analysed in a transmission electron microscope Zeiss EM-109 coupled to the image capture software MegaviewG2/Olympus Soft Imaging Solutions.

RNA Extraction, Reverse Transcription, and PCR Conditions

The polarised Caco-2 cells were infected with 2×10^7 CFU; a negative control was used. The experiment was performed in triplicate with two replications. At 15, 30 and 60 min and 2 and 4 h p.i., total cellular RNA was extracted using TRIZOL LS Reagent (Invitrogen). DNA-free RNA was transcribed to complementary DNA (cDNA) using 1 µg of total RNA (200 ng/µl), 10 U RNase inhibitor (Invitrogen), 40 U MMLV-RT (Amersham Biosciences), 1× MMLV-RT buffer (Amersham Biosciences), 200 µM dNTPs and 126 pmol random hexamer primers. Quantitative real-time PCR (qPCR) was performed using primers designed by us (Table 1). PCR mixtures (10 µl) contained 5 µl of *Master Mix SYBR® Green PCR Core Reagents* (Applied Biosystems) and 5 pmol of *primers*. The optimal annealing temperature for each primer pair is described in Table 1. Water was used as negative control. A single fluorescence measurement was taken after each thermo cycle. The specificity of the amplifications was verified by melting curve analyses and 2 % agarose gels. All primers yielded specific melting curves and electrophoresis bands as expected (data not shown). The efficiency of the amplification for each primer pair was assessed by the slope of the equation of a standard curve generated from the serial dilutions of the pooled samples.

Table 1 List of primers for each gene studied

Genes	Primers	Sequence 5' → 3'	Size (bp)	Annealing temperature T (°C)
CXCL-1	CXCL-1 forward	GTGGCCACTGAACTGCGC	146	58
	CXCL-1 reverse	GCTATGACTTCGGTTTGGGC		
IL8	IL8-forward	TGGAGAAGTTTTGAAGAGGGC	149	60
	IL8-reverse	GCATCTGGCAACCCTACAACA		
IL6	IL6-forward	TCAGCCCTGAGAAAGGAGACAT	76	58
	IL6-reverse	GTTTGTCTTCTGCCAGTGCC		
TGFβ-1	TGFβ-1 forward	AGTGGTTGAGCCGTGGAGG	53	58
	TGFβ-1 reverse	AGAAGCAGGAAAGGCCGGT		
B2M	B2M forward	AGCAGAGAATGGAAAGTCAAA		
	B2M reverse	TGTTGATGTTGGATAAGAGAA

Fig. 1 Quantification of *C. jejuni* inside Caco-2 cells

The Ct (“Cycle Threshold”) allows relative quantification of DNA of each sample. The Ct of the target gene was normalized by the Ct reference gene endogenous B2 M. For the relative quantification of transcript levels of genes under study (fold change), the treated group was compared to the control group by the method described by Livak and Schmittgen [18] with modifications. Thus, the Ct was normalized to the expression of endogenous using the formula dCt . Subtracting the value of dCt in the control group and the infected group provided the value of $ddCt$ and from there found the value of fold change for the derivation of the formula 2^{-ddCt} . For the negative results was applied the formula $-(2^{(ABS(ddCt))})$ and the positive results of $ddCt$ the formula 2^{ddCt} was utilized.

Statistical Analyses

We used the Kolmogorov-Sminorv to verify that the data were parametric. Obeyed the normal distribution, the data were analysed for statistical significance using one-way ANOVA and Tukey’s tests (Graphpad Prism 5). A p value ≤ 0.05 was considered significant. The graphs

Table 2 Quantification of *Campylobacter jejuni* (log CFU/mL) after cytochalasin-D treatment

	5 min	15 min	30 min	60 min	2 h	4 h
Positive control (mean)	Ab	4.00	4.00	4.69	4.41	4.34
Negative control	Ab	Ab	Ab	Ab	Ab	Ab
Cytochalasin treated 1	Ab	Ab	4.69	<4	<4	4.00
Cytochalasin treated 2	Ab	Ab	4.20	<4	<4	<4
Cytochalasin treated 3	Ab	Ab	Ab	<4	4.73	4.85

Ab absent, Neg negative, pos positive

Table 3 Quantification of *Campylobacter jejuni* (log CFU/mL) after nocodazol

	30 min	4 h
Positive control (mean)	4.12	4.22
Negative control	Ab	Ab
Nocodazol treated1	Ab	<4.00
Nocodazol treated 2	Ab	<4.00
Nocodazol treated 3	Ab	4.00

Ab absent, Neg negative, pos positive

were constructed based on the fold change value that shows how many times the infected group expresses the target gene compared to the control group (Graphpad Prism 5).

The data were analysed for statistical significance using one-way ANOVA and Tukey's tests (Graphpad Prism 5). A p value ≤ 0.05 was considered significant.

Results and Discussion

Campylobacter jejuni Invasion of Caco-2 Cells

Examination of bacterial internalisation in Caco-2 cells shows that the bacterium enter the cell 15 min p.i. with a peak internalisation 60 min. (Fig. 1). It is possible that intracellular bacterial multiplication is not as important of a mechanism for *C. jejuni* as it is for the *Salmonella* species [22, 29]. As real-time PCR was used in this study, it can be stated that the bacterium enters the cell, but it is not known if it remains viable throughout the period.

The negative findings in Caco-2 cells after treatment with nocodazole or cytochalasin indicates that actin and tubulin are involved in bacterial entry (Table 2 and 3). In the case of cytochalasin treatment, the results are consistent enough to state that after 15 min. p.i., there was involvement of actin filaments. After 30 min, one negative and two positive results were detected.

Fig. 2 TEM images of Caco-2 cells showing the internalisation of *C. jejuni* and the fusion with lysosomes. **A, B** The presence of bacteria (black arrow) near the tight junctions (white arrow) 15 and 60 minutes p.i. **C** The presence of internalised bacteria near tight junctions at 60 min p.i. **D** The fusion of lysosomes with bacteria and 15 min p.i. (cross). TEM images of Caco-2 cells showing the internalisation of *C. jejuni* and fusion with lysosomes. **E, F** The fusion of lysosomes with bacteria 15 min p.i. (cross). In photomicrograph **E** and **F** (increased magnification of **D**), it is possible see the bacteria (triangle) and lysosome marked with acid phosphatase (star). **G** and **H** Bacteria (black arrow) internalised (black arrow) in the perinuclear region (N nucleus) 60 min p.i.

Real-time PCR is a highly sensitive technique, and the presence of a positive group indicates that the participation of microtubules and actin are important for the process of internalisation for at least the early time periods. The involvement of microfilaments in the early periods that can be connected to the survival of the drug may decrease over time. Even in internalised bacteria, after 1 h, a value below 4 log CFU/ml is observed. Unfortunately, the software system used for this real-time PCR does not provide the exact number for values <4 log CFU/mL; as a result, we cannot calculate the statistical differences relative to the positive control.

Some authors have reported that internalisation of *C. jejuni* is only actin dependent [3, 16, 23]. Biswas et al. [1] reported that the process is both actin and microtubule dependent. In contrast, Russell and Blake [26] reported that neither actin nor tubulin is important in the invasion process. The microtubule-dependent invasion theory is gaining importance [10, 24]; however, the participation of the cytoskeleton appears to be strain dependent [4].

At 15 min. to 1 h p.i., *C. jejuni* bacteria were seen in contact with the microvilli (Figs. 2A,B) as determined by transmission electron microscopy (TEM) analysis. *C. jejuni* was inside the Caco-2 cell at 15 min. p.i. and was present as bacteria fused with lysosomes at a period of a few min to 2 h p.i. (Figs. 2D,E). At 4 h p.i., a few lysosomes were observed. The bacteria were adjacent to tight junctions (Fig. 2C) and associated with the perinuclear region (Figs. 2G,H). This result (perinuclear position) corroborates with Hu and Kopeck [10]. According to these authors, the bacterium moves to this region along microtubules. The position near the tight junctions was observed in the process of internalisation [2, 12, 19].

Some bacteria develop an avoidance system that ensures survival and replication of the parasite within different intracellular compartments in host cells. One example is the bacterium *Mycobacterium tuberculosis*, which blocks lysosomal fusion to the bacterial inclusions. In addition, *Listeria monocytogenes* escapes the phagocytic vesicles for replication in the cytoplasm [6], and *Coxiella burnetii* and *Salmonella* adapt to acidic compartments [6, 25].

Fig. 3 Morphometry (diameter) of Caco-2 cells infected or uninfected with *C. jejuni*

There was fusion of lysosomal compartments with the bacterial vacuole at 15 min. p.i. If the bacteria are eliminated by the lysosomes, as is likely with this pathogen, the toxins released are responsible for triggering the immune response. *C. jejuni* may use a similar mechanism as was described for *Salmonella* by Oh et al. [25] for adaptation to the lysosomal compartments.

The morphometric analysis of Caco-2 cells infected by *C. jejuni* identified an increase in diameter, as measured at 15 and 60 min. (Fig. 3). Morphometric changes in diameter were observed in infected cells. This anomaly has been described in the literature as mainly due to the protein CDT

[14, 28]. CDT causes a distensor effect that induces apoptosis and DNA fragmentation in cells [13]. Changes in the cell size induced by CDT constitute an important pathogenesis mechanism because the subsequent cell death (as identified by other authors) may compromise the intestinal permeability and facilitate the entry of bacteria into deeper layers of the intestinal mucosa.

Figure 4 shows that the bacteria induced the transcription of IL-8 and TGF- β 4 but not IL-6. The level of IL-8 transcripts increased gradually from 1 to 4 h p.i. There was an increase in IL-6 and CXCL in times of 60 min and 2 h respectively. However, this increase did not occur in subsequent periods. Hickey et al. [8] found that besides the process of invasion and adhesion, the release of IL-8 may be a consequence of the action of CDT. In this study, we found an increase in cell size that is likely due to CDT. The cytokine IL-8 activity is very important for the host response to *C. jejuni* [17]. Some authors propose that the secretion of this cytokine in response to *C. jejuni* is dependent on the dose, strain and type of cell [9, 20].

The increased IL-6 within 60 min was punctual. We have not studied the levels of transcripts for IL-6 in a subsequent period however; others have found an increase in IL-6 in dendritic cells in late periods (>24 h) [11]. This may be explained by the early timing of the cell culture. Because the importance of IL-6 as a pro-inflammatory cytokine appears to be concomitant with increased levels of IL-1 β [7], it may be that at later times, this cytokine was

Fig. 4 Quantification of cytokine and chemokine transcripts from Caco-2 cells. Caco-2 cells were inoculated with *C. jejuni*; RNA was isolated at various times post-inoculation (p.i.). The data are representative of three independent experiments

expressed. Friis et al. [5] found secreted IL-6 in infected Caco-2 cells in 24 periods.

The cytokine TGF β 1 showed high levels of transcripts only in the initial period p.i., with a decrease after 30 min. Members of the TGF- β family appear to have an essential role in regulating the immune response and are important for intestinal homeostasis [27]. The highest transcript expression levels of this cytokine at 15 min. may be an attempt to modulate the response to the bacteria. However, due to increased levels of pro-inflammatory IL-8 and CXCL-2, it is possible that such modulation by TGF- β 1 was not successful.

Conclusions

Campylobacter jejuni invades epithelial Caco-2 cells near the tight junctions and can be found in the perinuclear region. After entry, lysosomal compartments fuse with the vacuoles containing the bacteria and the entry of the bacteria causes an increase in cell size. In response, there is an increased cytokine transcripts that induces an inflammatory process.

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