Campylobacter jejuni induces diverse kinetics and profiles of cytokine genes in INT-407 cells

Ahlam I. Al-Amri, BSc, MSc, Giuseppe A. Botta, MD, PhD, Khaled S. Tabbara, PhD, Abdelrahman Y. Ismaeel, BSc, PhD, Ali E. Al-Mahmeed, BSc, MSc, Ahmed Y. Qareeballa, BSc, MSc, Khalid M. Bin Dayna, MSc, PhD, Moiz O. Bakhiet, MD, PhD.

ABSTRACT

الأهداف: تهدف الدراسة لاختبار القوى الحركية للخلايا الجنينية الظهارية البشرية على تعبير المرسل الوراثي لمختلف السيتوكينات والكيموكينات كاستجابة للسموم المفرزة من قبل بكتيريا الكمبيلوبكتر جيجني.

الطريقة: في دراسة تجريبية غير متحيزة تم حث الخلايا الجنينة الظهارية البشرية بواسطة السموم المفرزة من قبل بكتيريا الكمبيلوبكتر جيجني في المزرعة لفحص قدرتها على تعبير المرسل الوراثي لمختلف السيتوكينات والكيموكينات. ولقد تم استخدام التقنية المعدلة وهي التهجين الموقعي لتتبع ظهور المرسل الوراثي للسيتوكينات المحفزة للالتهابات إنترفيرون جاما، IL-1β, IL-6, IFN-γ, TNF-α, TGF-β1, and IL-8, and the anti-inflammatory cytokines IL-4 and IL-10.
أجريت هذه الدراسة في الفترة ما بين سبتمبر 2005م ومارس 2007م بقسم المايكروبيولوجي والمناعة والأمراض المعدية بكلية الطب، جامعة الخليج العربي، البحرين.

النتائج: عبرت الخلايا عن المرسل الوراثي للسيتوكينات المحفزة للالتهابات بعد معالجتها ببكتيريا الكمبيلوبكتر الحية مسجلة أعلى ارتفاع عند 12 ساعة منذ بدء الالتهاب. وعبرت عن المرسل الوراثي للسيتوكينات المضادة للالتهابات بعد معالجتها بالوسط البكتيري المرشح أو البكتيريا المكسرة بالموجات الصوتية وسجلت أعلى ارتفاع عند 3 ساعات منذ بدء الالتهاب. تم استخدام 4 عينات لكل سايتوكاين في كل وقت زمني محدد.

خاتمة: بينت الدراسة أن استجابة الخلايا الجنينية الظهارية لبكتيريا الكمبيلوبكتر جيجني له تأثير على المرسل الوراثي للسيتوكينات والكيموكينات وعلى وقت ظهوره. وهذا يدلنا على أن هنالك طريقة ما بواسطتها تقوم خلايا الجهاز الهضمي بإحداث وظيفة إفرازية، التهابية بالقولون بالتأثير على نفسها أو الخلايا المجاورة لها عن طريق إنتاج السيتوكينات مع/أو الكيموكينات خلال الالتهاب المعوي الأمر الذي قد يكون مؤثرا عند استنباط علاجات مناعية جديدة في حالة الالتهابات الحادة نتيجة عدوى بكتيرية.

Objectives: To examine the kinetic ability of embryonic human epithelial INT-407 cells to express messenger ribonucleic acid (mRNA) for various cytokines and chemokines in response to *Campylobacter jejuni (C. jejuni)* stimulation.

Methods: In an experimental single-blind study, cultured embryonic human epithelial INT-407 cells were treated with different concentrations of viable *C. jejuni*, its sonicated, and filtered supernatant. A modified non-radioactive in situ hybridization using probe cocktails was used to measure mRNA levels for the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, interferon-gamma (IFN-γ), tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, and IL-8, and the anti-inflammatory cytokines, IL-4 and IL-10. The study was carried out from September 2005 to March 2007 at the Department of Microbiology, Immunology, and Infectious Diseases, College of Medicine, Arabian Gulf University, Bahrain.

Results: Viable *C. jejuni*, sonicated bacteria and filtered supernatant induced high mRNA expression for the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, TNF-α, TGF-β1, and IL-8, which peaked at the 12 hours post stimulation. Anti-inflammatory cytokines IL-4 and IL-10 mRNA expression were induced maximally at 3 hours post stimulation mainly by sonicated bacteria and filtrated supernatant, however, not with living bacteria. Untreated embryonic human epithelial INT-407 cells expressed low amount of mRNA for the various cytokines and chemokines at all time points. For each cytokine, 4 samples were used per time hour.

Conclusion: This study demonstrated that embryonic human epithelial INT-407 cells in response to viable *C. jejuni* or its cytotoxins can alter cytokine and chemokine mRNA expression patterns and kinetics suggesting a potential role for theses mediators in the immunopathogenesis of the infection caused by this pathogen, which might be relevant for future immunotherapeutic interventions during severe bacterial infections.

Saudi Med J 2008; Vol. 29 (4): 514-519

From the Department of Molecular Medicine of Princess Al-Jawhara Center (Al-Amri, Bakhiet), Department of Microbiology, Immunology and Infectious Diseases (Al-Amri, Botta, Tabbara, Ismaeel, Al-Mahmeed, Qareeballa, Bin Dayna), College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Kingdom of Bahrain

Received 21st October 2007. Accepted 18th February 2008.

Address correspondence and reprint request to: Dr. Moiz Bakhiet, Professor and Chairman, Department of Molecular Medicine, College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Kingdom of Bahrain. Tel. +97 (317) 293664. E-mail: moiz@drmoiz.com

Campylobacter jejuni (C. jejuni) is considered as one of major causes of pediatric diarrhea in developing and well-developed countries.[1,2] The pathophysiology of diarrheal diseases and the severe long-term complications such as neurological damage due to the immune response of the host caused by *C. jejuni* is not fully understood,[3] although the adhesion, invasion, production of enterotoxin and cytotoxin have been reported to be the possible virulence factors.[4,5] Inflammation is a consequence of immune system activation that might be triggered by bacterial infections. While inflammation is an important element in the defense against infection, it can also contribute some complications such as Guillain-Barré syndrome, acute transverse myelitis, and neuropathies.[6-8] Therefore, the control of the immune and the inflammatory response of the gastrointestinal tract to bacteria is important in determining disease progression. Cytokines are very well known to play crucial roles in pathogenesis of inflammatory[9] and non-inflammatory disorders such as changes in cognitive functions and progression of the degenerative processes of aging.[10] Also, cytokines modify the functioning of epithelial cells. For instance, tumor necrosis factor (TNF-α) can modify intracellular signal transduction and regulate ionic channel activities in various epithelial cells.[11] Many epithelial cell lines can be invaded by *C. jejuni*, which damage the epithelium and act intracellularly.[12-15] We previously showed by in vitro experiments on INT-407 cells that *C. jejuni* can induce secretion of interleukin (IL)-6, interferon-gamma (IFN-γ), TNF-α, IL-4, IL-10, and IL-8 proteins, and mRNA expression for IL-8, and TNF-α.[16] In view of this, the current experiments were designed to study the de novo synthesis of these potential mediators in a kinetic manner to further explore their role during the disease process.

Methods. The study was carried out from September 2005 to March 2007 at the Department of Microbiology, Immunology, and Infectious Diseases, College of Medicine, Arabian Gulf University, Bahrain. This study received ethical approval from the Research and Ethics Committee of the College of Medicine and Medical Sciences, Arabian Gulf University, Bahrain.

Bacterial cultures and dilutions. One human strain was included in this study. It was isolated from a Bahraini child with dysentery. It was designated as Salmaniya Medical Complex 3915. The culture of the organism was carried out using conventional methodology for the isolation of *Campylobacter*.[17] *Campylobacter jejuni* was adjusted to 10^6 cells/ml (titration studies showed that this concentration of bacteria is optimum to stimulate cytokines and chemokines).

Preparation of sonicated C. jejuni. *Campylobacter jejuni* cultures grown for 48 hours on 3x100mm diameter plates were harvested into one ml of Eagle's minimum essential medium (EMEM). *Campylobacter jejuni* of 10^8 cells/ml were disrupted by 6x30 second pulses on ice with a VirSonic 50 Sonicator (Virtis, Gardiner, New York). The sonicated bacteria were diluted in EMEM and adjusted to 1:10 dilution.

Preparation of C. jejuni supernatant filtrates. Pure *C. jejuni* cultures grown for 48 hours were harvested into one ml of phosphate buffer solution (PBS) to a suspension of 10^8 cells/ml and centrifuged at 14,000 x g at 4°C for 20 minutes. The supernatant was removed and filtered through 0.45 μm Nalgene filter. The filtrate, which was free of bacterial cells, was diluted with PBS to 1:10.

Cell cultures. The human embryonic intestinal epithelial cell line INT-407 was used in this study (Hyper Cell Line Data Base, Public Health, United Kingdom). Morphology and mucin secretion were tested, and these cells were found to retain the normal phenotype. The cells were fed with EMEM supplemented with 5% fetal bovine serum and 0.5% L-glutamine (Gibco, Gaithersburg, Maryland, USA). The cells were grown to a polarized monolayer in an 80 cm flask, washed, and released with trypsin-ethylenediaminetetra acetic acid and plated onto Lab-Teck 4 chambers slides (NUNC brand, Rochester, New York). After the polarized INT-407 cells were formed, they were infected with 10^6 viable bacterial cells (approximately 41.6 bacteria/cell), or treated with crude extract after sonication or culture filtrates in dilution of 1:10. Cytokines messenger ribonucleic acid (mRNAs) were detected at 1, 3, 12, and 24 hours following treatment using the in situ hybridization, leaving some wells without stimulation as negative controls. For each cytokine, 4 samples were used per time hour (**Figures 2 & 3**).

Preparation of INT-407 polarized monolayers for in situ hybridization. For detection of IL-1β, IL-6, IFN-γ, TNF-α, IL-4, IL-10, transforming growth factor (TGF)-β1, and IL-8 mRNA after the previously mentioned treatment times, INT-407 cells were washed with diethylpyrocarbonate solution/PBS. After pre-treatment with 0.4 μg/ml Proteinase K+Tris, cell structures were preserved with 0.4% paraformaldehyde in 1x PBS, INT-407 cells were hybridized (Research and Dignostic Systems, South Africa) with Biotin labeled antisense RNA probe cocktail (200 ng/ml,

Disclosure: This work was supported by a grant from the College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Bahrain.

R&D, SA). Hybridization was performed overnight at 37°C. After hybridization, the polarized monolayers were washed with DEPC/PBS and fixed with methanol. The monolayers were washed with decreasing grades of sodium chloride/sodium citrate buffer and permeabilized with 1x PBS/Triton.

Biotin labelled mRNA detection. The monolayers were stained using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), and slides were counterstained with hematoxylin.

Digoxigenin labelled mRNA detection. The de novo expressed mRNAs were fixed with the DAKO Rabbit F (ab') Anti-digoxigenin/horse redish peroxide in dilution of 1:100, and then liquid 3,3'-Diaminobenzidine-Nitro Blue Tetrazolium-Chloride-Chromogen solution was added (DAKO corp., Carpinteria, CA, USA), and slides were counterstained with hematoxylin. The in situ hybridized cells were examined in a Leica RXM microscope (Leica, Wetzlar, Germany) equipped with a 3DD color camera (Sony, Tokyo, Japan). Enumeration of cytokine mRNA expressing cells was performed manually at X 630 original magnification.

Statistical analysis. Analysis of variance (ANOVA) was used for multiple comparisons. Student's unpaired t-test was used to measure statistical significance between selected 2 groups (experimental versus non-stimulated control). The significance of cytokine expression on normal INT-407 was compared with those infected with live *C. jejuni*. Thereafter, cytokine expressions on INT-407 cells infected with sonicated as well as supernatant derivatives of the organism were compared. Stars indicate level of significance ($*p<0.01$, $**p<0.001$, $***p<0.0001$). We used INSTAT2 for our statistical analysis.

Results. A qualitative difference in mRNA signal (staining) was noted depending on the period of stimulation. All cells expressing mRNA, regardless of strength of signal were considered positive. Viable *C. jejuni*, sonicated bacteria and filtered supernatant induced high mRNA expression for the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, TNF-α, TGF-β1, and IL-8, which peaked at 12 hours post stimulation. Anti-inflammatory cytokines IL-4 and IL-10 mRNA expression were induced maximally at 3 hours post stimulation mainly by sonicated bacteria and filtrated supernatant, however, not with living bacteria. Untreated embryonic human epithelial INT-407 cells expressed low amounts of mRNA for the various cytokines and chemokines at all time points. To illustrate these differences, photomicrographs of IL-1b

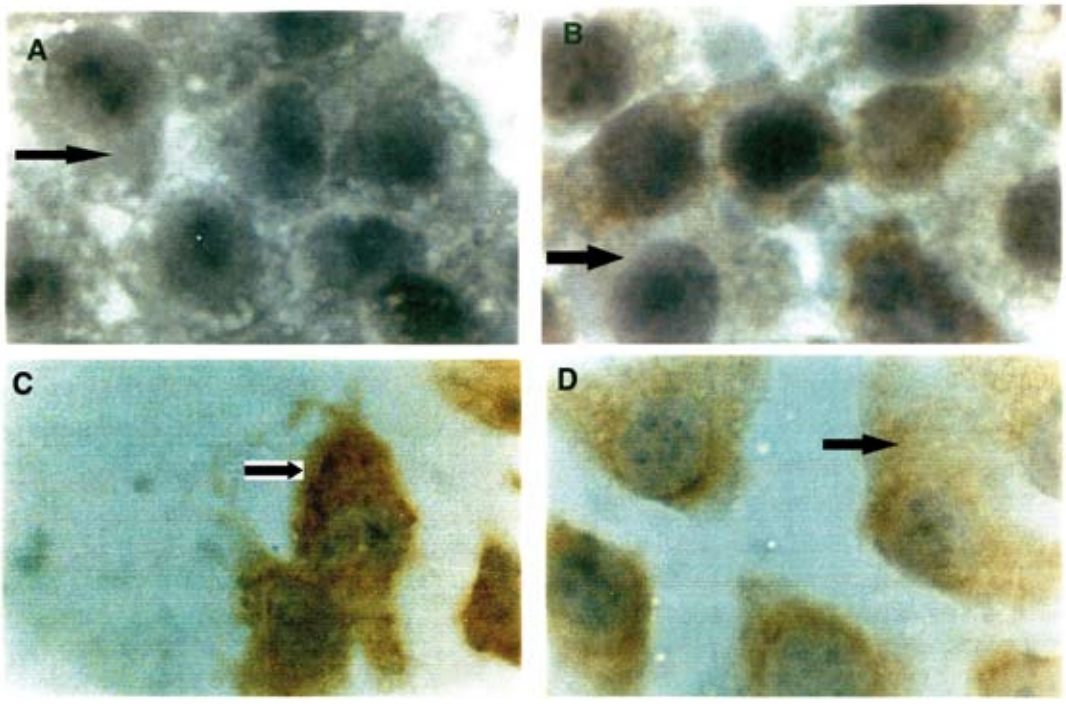

Figure 1 - Interleukin (IL)-1β messenger ribonucleic acid (mRNA) expression as an example for cytokine/chemokine gene expression as detected by in situ hybridization in INT-407 cells infected with 10^6 *Campylobacter jejuni* at: **a**) 0 hour (negative control), **b**) 3 hours, **c**) 12 hours, and **d**) 24 hours. The arrow on **c**) shows maximum intracellular staining indicating peak of IL-1β mRNA expression at 12 hours. Pictures were taken in bright field microscopy (x 200).

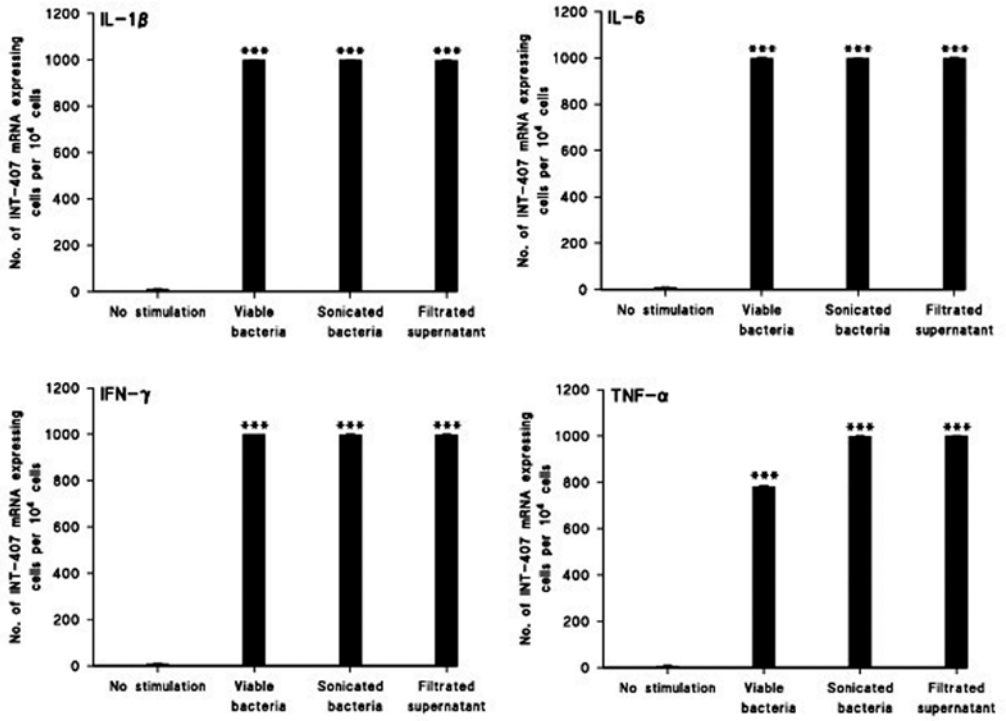

Figure 2 - The figure demonstrates numbers of cytokine messenger ribonucleic acid (mRNA) expression by INT-407 cells for the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF-α), and interferon-gamma (IFN-γ). The human embryonic intestinal epithelial cell line INT-407 was used in this study. For each cytokine 4 samples were used per time hour. The study was repeated 3 times with similar results (n=8). Means ± standard deviation are shown, (***p<0.0001).

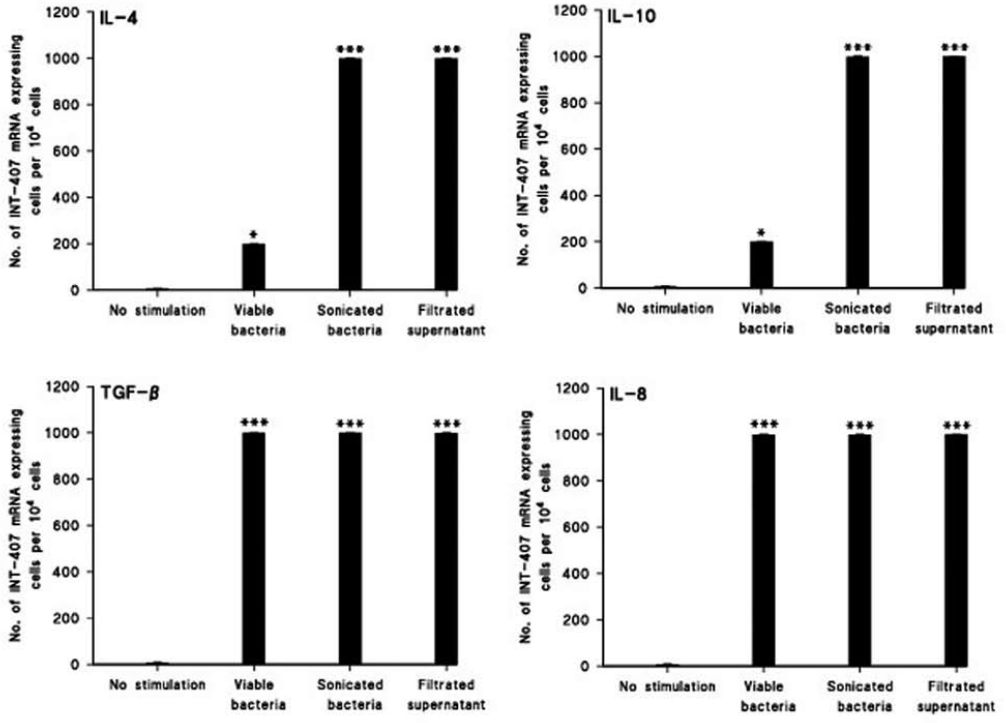

Figure 3 - The figure demonstrates cytokine messenger ribonucleic acid (mRNA) expression by INT-407 cells for the anti-inflammatory cytokines interleukin (IL)-4, IL-10, transforming growth factor (TGF-β) and the chemokine IL-8. The human embryonic intestinal epithelial cell line INT-407 was used in this study. For each cytokine 4 samples were used per time hour. The study was repeated 3 times with similar results (n=8). Means ± SD are shown, (*p<0.01, ***p<0.0001).

mRNA expression (as an example of pro-inflammatory cytokines) by human intestinal epithelial INT-407 cells stimulated by infection with *C. jejuni* for 0, 3, 12, and 24 hours are shown in **Figure 1**.

Each cytokine showed different kinetics after stimulation with viable *C. jejuni*, sonicated *C. jejuni*, or *C. jejuni* filtered supernatants. The pro-inflammatory cytokines IFN-γ, IL-6, IL-1β, TGF-β1, TNF-α, and a potential chemokine IL-8 showed the maximum mRNA expression when the INT-407 cell were infected with 10^6 viable *C. jejuni*, peaking at 12 hours post stimulation. For IL-1β, IL-6, IFN-γ, TNF-α, TGF-β1 and IL8, more than 10^3 cells per 10^4 expressed mRNA for these cytokines after stimulation with viable *C. jejuni*, sonicated *C. jejuni*, or *C. jejuni* filtered supernatants (except TNF-α stimulated with viable bacteria where approximately 800 cells per 10^4 expressed the mRNA without affecting the level of significance) (**Figures 2 & 3**). The anti-inflammatory cytokines IL-4 and IL-10 showed the maximum mRNA expression when the INT-407 cell were treated with a 1:10 dilution of sonicated *C. jejuni* as well as *C. jejuni* filtered supernatants, peaking at 3 hours following treatment. Those anti-inflammatory cytokines were highly induced by sonicated bacteria and filtrated supernatant where more than 10^3 cells per 10^4 expressed mRNA for these cytokines, however, not with living bacteria since only 200 cells per 10^4 expressed the mRNA for IL-4 and IL-10 (**Figure 3**). Untreated INT-407 cells expressed pro-inflammatory and anti-inflammatory cytokines and chemokines at very low levels as shown in **Figures 2 & 3**.

Discussion. The INT-407 cells were used in this study as an in vitro model system for intestinal epithelium to measure the endogenous expression of cytokine/chemokine by the colonic epithelial cells. After treating the cells with viable *C. jejuni*, the mRNA levels for the pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ, and TNF-α) and the chemokine (IL-8) peaked at 12 hours post infection. While treating the same cells with sonicated *C. jejuni* or filtrated supernatant, the anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) peaked at 3 hours post stimulation. However, living bacteria induced low levels of cytokine mRNA for IL-4 and IL-10 and high level for TGF-β. These results suggest that the intestinal epithelial cells can modify their own cytokine and chemokine expression after bacterial challenge at the mRNA level to deal with infection. Previous work has shown that cytokine mRNA expression peaked at 4-8 hours and protein production to peak at 24-48 hours[16,18-23] and coupled with this work, suggesting that the production of these cytokines is under transcriptional control. The protein production of these cytokines and chemokine along with the precise nature of the interaction between *C. jejuni* and cell surface receptors warrant further study.

In this study, viable *C. jejuni* stimulated the mRNA expression for the anti-inflammatory cytokine TGF-β1 at a high level suggesting that this cytokine is present in active form,[24] and mRNA for TGF-β1 is expressed by the regulatory T helper (Th) cells in vivo and it acts upon Th1 and Th2 cells.[25] This suggests the association between the presence of cytolethal distending toxin (CDT) and other cytotoxins in the sonicated *C. jejuni* and its filtered supernatant, which need the cooperation of both Th1 and Th2 cells responses.[25] Our data showed that the INT-407 cells stimulated with sonicated *C. jejuni*, or its filtered supernatant expressed anti-inflammatory cytokine mRNA suggesting that the 5 cytotoxines[26] influenced the expression of cytokines needed for B-cell activation and hence antibody secretion. Antibodies play a role in neutralization of soluble bacterial toxins.[25]

Also, the data of this study showed a balance between Th1 and Th2 cell responses represents a sort of switch, which can be used to bias immune response in one or another direction. The result of this differentiation switch is to activate 2 different pathways of immunity, which is associated with different immune responses. The Th1 pathway is essentially cell- mediated immunity necessary to eliminate intracellular antigens, which was the case when stimulating with viable cytolethal destending toxin producing *C. jejuni*. The protective immune response to soluble cytotoxins present in the sonicated *C. jejuni* or its filtered supernatant would be dominated by an antibody response, which fits with a Th2 response.

Detection of mRNA is essential to depict de novo synthesis of protein. Therefore, mRNA expression with protein production should be very well correlated for the normal function of the cell.[27] The mRNA expression results are almost certainly only correlative, instead of causative,[28,29] in the end it is, most probably, the concentration of proteins and their interactions that are the true causative forces in the cell, and it's the corresponding protein quantities that we should be looking at.

In conclusion, the present data are useful for defining the expression of mRNA and related kinetics for key cytokines in response to INT-407 *C. jejuni* stimuli. Large-scale mRNA expression measurements raise the possibility of extracting information on the underlying genetic regulatory interactions directly from the data. This suggests a mechanism whereby human intestinal epithelial cells in vivo may mediate colon secretory function, inflammation, and diseases in an autocrine/paracrine fashion by expressing, producing, and secreting cytokines and chemokines during gastroenteritis.

References

1. O'Ryan M, Prado V, Pickering LK. A millennium update on pediatric diarrheal illness in the developing world. ***Semin Pediatr Infect Dis*** 2005; 16: 125-136.
2. Richardson G, Thomas DR, Smith RM, Nehaul L, Ribeiro CD, Brown AG, et al. A community outbreak of *Campylobacter jejuni* infection from a chlorinated public water supply. ***Epidemiol Infect*** 2007; 135: 1151-1158.
3. Wassenaar TM, Blaser MJ. Pathophysiology of *Campylobacter jejuni* infections of humans. ***Microbes Infect*** 1999; 1: 1023-1033.
4. Al-Shaikh SA, Senok AC, Ismaeel AY, Botta GA. Invasive capabilities of *Campylobacter jejuni* strains isolated in Bahrain: molecular and phenotypic characterization. ***Acta Microbiol Immunol Hung*** 2007; 54: 139-150.
5. Konkel ME, Joens LA. Adhesion to and invasion of Hep-2 cells by *Campylobacter spp.* ***Infect Immun*** 1989; 57: 2984-2990.
6. Yuki N, Kuwabara S. Axonal Guillain-Barré syndrome: carbohydrate mimicry and pathophysiology. ***J Peripher Nerv Syst*** 2007; 12: 238-249.
7. Baar I, Jacobs BC, Govers N, Jorens PG, Parizel PM, Cras P. *Campylobacter jejuni*-induced acute transverse myelitis. ***Spinal Cord*** 2007; 45: 690-694.
8. Kimoto K, Koga M, Odaka M, Hirata K, Takahashi M, Li J, et al. Relationship of bacterial strains to clinical syndromes of *Campylobacter*-associated neuropathies. ***Neurology*** 2006; 67: 1837-1843.
9. Tayal V, Kalra BS. Cytokines and anti-cytokines as therapeutics - An update. ***Eur J Pharmacol*** 2008; 579: 1-12.
10. Dinarello CA. Historical insights into cytokines. ***Eur J Immunol*** 2007; 37: S34-S45.
11. Luster AD. Chemokines-chemotactic cytokines that mediate inflammation. ***New Engl J Med*** 1998; 338: 436-445.
12. Papadakis KA, Targan SR. Tumour necrosis factor: biology and therapeutic inhibitors. ***Gastroenterology*** 2000; 119: 1148-1157.
13. Brás AM, Ketley JM. Transcellular translocation of Campylobacter jejuni across human polarised epithelial monolayers. ***FEMS Microbiol Lett*** 1999; 179: 209-215.
14. Harvey P, Battle T, Leach S. Different invasion phenotypes of *Campylobacter* isolates in Caco-2 cell monolayers. ***J Med Microbiol*** 1999; 48: 461-469.
15. Russel RG, Blake DC. Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. ***Infect Immun*** 1994; 62: 3773-3779.
16. AL-Salloom FS, AL-Mahmeed A, Ismaeel A, Botta GA, Bakhiet M. *Campylobacter*-stimulated INT407 cells produce dissociated cytokine profiles. ***J Infect*** 2003; 47: 217-224.
17. MacCallum AJ, Harris D, Haddock G, Everest PH. *Campylobacter jejuni*-infected human epithelial cell lines vary in their ability to secrete interleukin-8 compared to in vitro-infected primary human intestinal tissue. ***Microbiology*** 2006; 152: 3661-3665.
18. Esnault S, Benbernou N, Lavaud F, Shin HC, Potron G, Guenounou M. Differential spontaneous expression of mRNA for IL-4, IL-10, IL-13, IL-2 and interferon-gamma (IFN-gamma) in peripheral blood mononuclear cells (PBMC) from atopic patients. ***Clin Exp Immunol*** 1996; 103: 111-118.
19. Fan J, Nishanian P, Breen EC, McDonald M, Fahey JL. Cytokine gene expression in normal human lymphocytes in response to stimulation. ***Clin Diagn Lab Immunol*** 1998; 5: 335-340.
20. Heijligenberg R, Romijn JA, Godfried MH, Endert E, Sauerwein HP. In vitro production of cytokines in whole blood versus plasma concentrations of cytokines in AIDS. ***AIDS Res Hum Retroviruses*** 1998; 14: 123-127.
21. Hyjek E, Lischner HW, Hyslop T, Bartkowiak J, Kubin M, Trinchieri G, et al. Cytokine patterns during progression to AIDS in children with perinatal HIV infection. ***J Immunol*** 1995; 155: 4060-4071.
22. O'Mahony L, Holland J, Jackson J, Feighery C, Hennessy TP, Mealy K. Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. ***Clin Exp Immunol*** 1998; 113: 213-219.
23. Than S, Hu R, Oyaizu N, Romano J, Wang XP, Sheikh S, et al. Cytokine pattern in relation to disease progression in human immunodeficiency disease virus-infected children. ***J Infect Dis*** 1997; 175: 47-56.
24. Mire-Sluis AR, Thorpe R. Laboratory protocols for the quantitation of cytokines by bioassay using cytokine responsive cell lines. ***J Immunol Methods*** 1998; 211: 199-210.
25. Goldspy R, Kindt TJ, Osborne BA. Cytokines. In: Goldspy R, Kindt TJ, Osborne BA, editors. Kuppy Immunology. New York (NY): WH Freeman Company; 2000. p. 308-311.
26. Wassenaar TM, Bleumink-Pluym NM, van der Zeijst BA. Inactivation of Campylobacter jejuni flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. ***EMBO J*** 1991; 10: 2055-2061.
27. Hatzimanikatis V, Choe LH, Lee KH. Proteomics: theoretical and experimental considerations. ***Biotechnol Prog*** 1999; 15: 312-318.
28. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. ***Science*** 1999; 286: 531-537.
29. Macgregor PF, Squire JA. Application of microarrays to the analysis of gene expression in cancer. ***Clin Chem*** 2002; 48: 1170-1177.