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Effect of *Vibrio cholerae* on Chemokine Gene Expression in HT29 cells and its Modulation by *Lactobacillus* GG

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Abstract

Epithelial cells participate in the innate immune response to pathogenic bacteria by elaborating chemokines. This study examined the effect of *Vibrio cholerae* and *Lactobacillus rhamnosus* GG on inflammatory chemokine gene expression in the HT29 human intestinal epithelial cell line. HT29 cells were exposed to *V. cholerae* 0139, *Lactobacillus* or both for 2 h and cultured further thereafter for 4 h. RNA was extracted from the cells and expression of genes for chemokines and related molecules was quantitated by real time PCR using a pathway-focused PCR array. TLR4 was silenced using shRNA and output of interleukin-8 (IL-8) into the media quantitated with and without *V. cholerae* exposure. NFκB and p38 MAP kinase activation were determined by immunoblotting for IκBα and phosphorylated p38. *Vibrio cholerae* significantly upregulated gene expression for the neutrophil chemoattractant CXCL chemokines, IL-8, CXCL and CXCL in HT29 cells, while downregulating the expression of macrophage-attracting C-C chemokines. TLR4 silencing did not reduce IL-8 output from HT29 cells in response to *V. cholerae*. IκBα degradation was noted in the HT29 cells soon after exposure to *V. cholerae* and this recovered over time after removal of bacteria. p38 MAP kinase activation was not noted. *Vibrio cholerae* upregulated the expression of neutrophil attractant chemokines, most prominently IL-8, in HT29 cells, but downregulated macrophage-attracting chemokines. Probiotic lactobacilli modulated the IL-8, but not the other chemokine gene changes, in response to *V. cholerae*.

Introduction

Cholera, caused by the bacterium *Vibrio cholerae* remains a significant public health problem characterized by pandemics originating from areas of endemicity [1]. *Vibrio cholerae* induces diarrhoea through the elaboration of cholera toxin which stimulates adenyl cyclase in intestinal epithelial cells and leads to fluid secretion [2]. Although considered to be the paradigm of non-inflammatory secretory diarrhoea, increases in inflammatory factors such as myeloperoxidase, lactoferrin, nitric oxide and eicosanoids, are reported in cholera [3]. The presence of congested blood vessels and polymorphonuclear leucocyte infiltration of the lamina propria have been reported in the acute stage of cholera in both adults and children [4]. Polymorphonuclear leucocytes can induce chloride secretion similar to that seen with enterotoxins [5] and this could play an important role in diarrhoea. Epithelial cells secrete interleukin-8 (IL-8), a potent chemokine and chemoattractant of polymorphonuclear leucocytes, when they contact pathogenic bacteria [6]. Chemokine secretion is probably not dependent on the presence of cholera toxin, as volunteers given toxin-deficient strains also developed significantly increased titres of lactoferrin and CXCL8 [7]. Chemokines are subdivided into four subfamilies on the basis of the position of either one or two cysteine residues located near the amino terminus of the protein (CXCL, CCL, CL and CX3CL) [8]. Studies in mice suggest that *V. cholerae* deficient in toxin production can elicit a major pro-inflammatory response [9]. The pro-inflammatory response induced by the bacterium is probably responsible for containment of the infection. Inoculation of *Vibrio* into mice with a mutation in Toll-like receptor 4 results in more severe disease and in attenuation of the cytokine responses, suggesting that the pro-inflammatory response is mediated at least in part by TLR4 [9].

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Probiotic bacteria are commonly used in the treatment of diarrhoeal disease. *Lactobacillus rhamnosus* GG (LGG), one of the best-studied probiotic bacteria, has been shown to be effective in the treatment of acute diarrhoea in children [10]. In the present study, we sought to study the pattern of activation by *V. cholerae* of chemokines in intestinal epithelial cells, and to determine whether *Lactobacillus* GG would modulate these innate inflammatory responses. To this purpose we used an inflammatory cytokine pathway-focused PCR array and also examined the role of TLR4 and of NFκB and p38 MAP kinase activation in the *Vibrio*-epithelial cell interaction.

Materials and methods

Cell culture. HT29 cells (National Center for Cell Sciences, Pune, India) were grown in culture flasks in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Paisley, UK) containing 100 U of penicillin/ml, 100 μg of streptomycin/ml and 20 μg of amphotericin B supplemented with 10% fetal bovine serum (Gibco BRL) and incubated at 37 °C with 5% CO2.

Bacterial protocols. A clinical isolate of *V. cholerae* O139, kindly provided by the Clinical Microbiology Department, Christian Medical College, Vellore, was used for these studies. Disease with either the O139 strain or the O1 strain is known to produce equivalent increases in inflammatory mediators [4]. *Lactobacillus* GG (Culturelle) was grown in de Man Rogosa Sharpe broth (Himedia, Mumbai, India) under anaerobic conditions for 16 h at 37 °C on the day before the experiments.

Bacterial culture and infection of HT29 cells. Bacteria were grown standing overnight at 37 °C in Luria–Bertani broth before use. They were pelleted by centrifugation at 800 *g* and washed three times with sterile phosphate-buffered saline (PH 7.4). The bacterial concentration in the suspension medium was determined by measurement of absorbance at 540–600 nm and comparing with standard barium chloride optical density. The bacteria were then re-suspended in antibiotic free medium.

HT29 cells were cultured in 24-well plates (Falcon Milliwell; BD, Franklin Lakes, NJ, USA) until they reached confluence. Prior to infection, cells were washed twice with appropriate medium without serum and antibiotics and maintained in serum free medium for at least 2 h. Following this, cells were overlaid with medium alone (control), *V. cholerae* (100 bacteria per epithelial cell) and/or *Lactobacillus* GG (1000 bacteria per epithelial cell) for 2 h. The ratios of bacteria per epithelial cell were determined in earlier studies examining the dose effect. After 2 h, the extracellular bacteria were removed by washing and further incubated for 4 h in the presence of 50 μg/ml gentamicin to kill the remaining extracellular bacteria. At the end of this period, culture supernatant was removed and stored at −20 °C for quantitation of IL-8 secretion, while cells were removed and stored at −80 °C for total RNA isolation.

ELISA for IL-8. Interleukin-8 was assayed in culture supernatants using ELISA (BD Opt EIA SET, Catalog No. 555, BD Biosciences, San Jose, CA, USA) after centrifugation at 9000 *g* for 10 min to remove bacterial debris. Assays followed the standard protocol recommended by the manufacturer. Substrate solution tetramethyl benzidine/H_20_2 (BD Biosciences) was added for colour development and plates were read at 450 nm in an ELISA plate reader (Victor 3; Perkin Elmer, Waltham, MA, USA).

Chemokine and chemokine receptors array. Total RNA was isolated from infected cells using Trizol reagent (Sigma, St Louis, MO, USA, Cat. No. T9424) as per manufacturer's instructions. Reverse transcription of RNA was performed in a final volume of 20 μl containing 0.5 mM of each nucleotide triphosphate, 40 U of RNAase inhibitor (Ambion, Foster City, CA, USA), 50–100 ng of random hexamers (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK), 200 U of Moloney Murine Leukaemia virus RT (Finnzymes) and 15 μl of the extracted RNA or H_20. The sample was then incubated at 42 °C for 1 h. The Human Chemokines and Receptors RT^2 Profiler™ PCR Array (SuperArray Inc., Frederick, MD, USA) was used for these studies. This is an inflammatory pathway-focused array containing primers targeting members of the C-C and C-X-C motif subfamilies of small inducible cytokines and their receptors as well as other related genes. The array profiles the expression of 84 genes that encode chemokines and their receptors (Table 1). In addition, it profiles five

Table 1 List of gene products profiled by the inflammatory chemokine pathway-specific PCR array.

Family	Genes involved
CC motif ligands	CCL1, CCL11, CCL13, CCL15, CCL16, CCL17, CCL18, CCL19, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8.
C-X-C motif ligands	CXCL1, CXCL10, CXCL11, CXCL12, CXCL13, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9.
CC motif receptors:	CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR10, CCRL1, CCRL2.
C-X-C motif receptors	BLR1, CXCR3, CXCR4, CXCR6, CYFIP2.
Other chemokines and related genes	AGTRL1, BDNF, C5, C5R1 (GPR77), CCBP2, CKLF, CKLFSF1, CKLFSF2, CKLFSF3, CKLFSF4, CMKLR1, CSF3, CX3CL1, CX3CR1, ECGF1, GDF5, GPR31, GPR77, GPR81, HIF1A, IL-13, IL-16, IL-18, IL-1A, IL-4, IL-8, IL-8RA, LTB4R, MMP2, MMP7, MYD88, NFKB1, SCYE1, SDF2, SLIT2, TCP10, TLR2, TLR4, TNF, TNFRSF1A, TNFSF14, TREM1, VHL, XCL1, XCR1.

housekeeping genes and two negative controls for comparison. The cDNA derived from the infected cell monolayers was diluted and quantitative PCR was performed as per manufacturer's instruction manual (www.SABiosciences.com) on a Chromo4 (Bio-Rad, Richmond, CA, USA) real time PCR system.

Melting curve analysis was performed for each well in the entire plate after the PCR to check the quality of the PCR products. Amplifications showing more than one peak in each reaction at temperatures greater than 80 °C were not considered for analysis in order to avoid non-specific amplification. After the qPCR, agarose (2%) gel electrophoresis was undertaken using 10 μl of each PCR product in order to confirm a single band product, confirmed against a 500 bp DNA ladder. Bands were documented using a gel documentation system (ChemiSmart, Vilber Lourmat, France).

Expression of the chemokine and related gene products was normalized against five housekeeping genes 18s rRNA, HPRT1, RPL13A, GAPDH and β-actin in each PCR array plate. mRNA expression was calculated relative to the housekeeping genes as suggested by the manufacturer. The fold-change for each gene was calculated as $2\text{^}(-\Delta\Delta Ct)$, and shown as upregulation if expression was significantly greater than 1, or downregulation if significantly less than 1.

TLR4 silencing in HT29 cells. In order to test the effect of TLR4 signalling on changes in chemokine gene expression induced by *V. cholerae*, TLR4 was silenced in HT29 cells using Toll-like receptor 4 Mission^{TM} shRNA lentiviral transducing particles (Cat No. SHVRS-NM-003266, Sigma-Aldrich). HT29 cells were seeded in 24 well plates (BD Biosciences) the day prior to infection and incubated overnight (37 °C, 5% CO_2). Next day the media was removed and fresh growth media containing hexadimethrine bromide (Polybrene, final concentration 8 μg/ml, H9268, Sigma, India) was added. The lentiviral shRNA or control transduction particles (Mission^{TM} SHC001V, PLKO.1-Puro, Sigma) were added to the cells, and incubated for 20 h (37 °C, 5% CO_2). Four TLR 4 shRNA constructs TRCN0000056893 (Clone ID: NM_003266.2-2519s1c1) TRCN0000056894 (Clone ID: NM_003266.2-592s1c1) TRCN0000056895 (Clone ID: NM_003266.2-1056s1c1) TRCN0000056896 (Clone ID: NM_003266.2-2728s1c1 – were used. After 20 h, the medium was changed and fresh medium containing puromycin was used in order to select stable cell lines expressing the shRNA. Puromycin concentration was optimized for each HT29 cell line, typical concentrations ranging from 2–5 μg/ml. Further incubation was done with media changes every few days. Incubation periods were highly dependent on the post-infection assay. Puromycin selection required at least 48 h.

Studies for NFκB activation and p38 MAP kinase activation. These signalling studies were done in HT29 cells infected with *V. cholerae* for time periods of 0, 0.5, 1 and 2 h. The infected cells were washed with ice cold PBS and then subjected to cytosolic and nuclear extract preparation (Nuclear extraction kit, Cat No.2900, Chemicon International, Temecula, CA, USA) as described in the manufacturer's protocol. The cytosolic portion and the nuclear extract were stored at −80 °C until use. The protein concentration in the entire sample was estimated by Lowry's method.

Proteins were separated by 10.0 and 12.0% SDS-polyacrylamide gel electrophoresis. They were then transferred for 1.5 h at 400 mA in transfer buffer (25 mM Tris-base, 150 mM glycine and 10% methanol) onto a polyvinylidene difluoride membrane (Millipore, India). Membranes were blocked overnight at 4 °C with 5% skim milk for IκB-α, GAPDH and TLR4, and then incubated with primary antibody for 2 h at room temperature. p38 phosphorylated and non-phosphorylated MAP kinase blots were blocked with 5% BSA for 2 h and incubated overnight in primary antibody. The antibodies used were as follows: anti-IκBα (1:3000, rabbit monoclonal; Abcam 32518, Abcam, Cambridge, UK), anti-p38 (1:2000, rabbit monoclonal; Cat. No. 9212, Cell Signaling, Danvers, MA, USA), anti-phospho-p38 (1:1000, mouse; Cat. No. 9216, Cell Signaling) and anti-GAPDH (1:3000 mouse monoclonal, mAbcam 9484, Abcam, UK). Secondary staining was conducted with HRP-conjugated goat sera specific for mouse or rabbit Ig (Cat. No. 1858415, 1858413, 1:1000, ECL Pierce, Rockford, IL, USA), followed by chemiluminescent detection in a ChemiSmart gel documentation apparatus (Vilber-Lourmat) after developing with a commercial reagent (Super Signal West Femto Maximum Sensitivity Substrate, Cat. No, 34095, Pierce) following manufacturer's instructions. Comparisons were made only among samples isolated and transferred together onto the same membrane. Multiple exposures were done to ensure that film was not overexposed. To confirm equal loading of protein, all Western blots using phospho-specific antibodies were stripped and reprobed with antibody against the non-phosphorylated kinase and GAPDH as a housekeeping gene.

Statistical analysis. All data were expressed as mean (SEM). Significance of differences between groups was assessed using the student *t*-test or Mann–Whitney independent test depending on the nature of distribution of the data. Two-tailed *P*-values <0.05 were considered statistically significant. The chemokine gene expression was analysed using the software available with the pathway focused array.

Results

Vibrio cholerae affected the gene transcription of several chemokine and related molecules, upregulating some and downregulating others (Table 2).

Table 2 Changes (upregulation or downregulation) in expression of important chemokine gene groups brought about in HT29 cells by infection with *Vibrio cholerae*.

		Fold up- or down-regulation		*P*-value	
Gene		*Lactobacillus*	*Vibrio cholerae*	*Lactobacillus*	*Vibrio cholerae*
CXC ligands					
CXCL8	IL-8	1.87	42.3	0.3802	0.015
CXCL10	IP-10	3.87	16.07	0.766	0.013
CXCL1	GRO-α	1.08	14.55	0.2859	0.03
CXCL5	ENA-78	1.16	−4.77	0.2439	0.0209
CC ligands					
CCL1	I-309	−1	−2.96	0.201	0.0122
CCL2	MCP-1	1.52	−2.98	0.2227	0.0096
CCL8	MCP-2	1.88	−2.71	0.4433	0.0514
CCL13	MCP-4	1.67	−3.18	0.3545	0.0238
CCL3	MIP-1 α	1.67	−3.13	0.326	0.0151
CCL4	MIP-1β	1.08	−3.87	0.2745	0.0299
CC receptor					
CCR8		−1.02	−3.73	0.2494	0.0358
CCR3		−1.2	−2.73	0.205	0.0584
Related genes					
TNF		3.08	6.17	0.6047	0.011
SLIT2		2.09	−2.8	0.4494	0.0653
TLR2		1.23	−3.2	0.3204	0.0739
TREM1		1.66	−3.33	0.3644	0.0388
TCP10		1.52	−3.39	0.3061	0.0292
MMP-7		1.97	4.65	0.3225	0.01

GRO, growth related oncogene; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor; ENA, epithelial neutrophil activated peptide.
Values represent mean of three independent experiments.
P-values were calculated using paired *t*-tests.
Results are shown only for the differences that were statistically significant or had a *P*-value approaching significance.

CXC motif ligands

Several members of this group, including IL-8, CXCL-10 and CXCL-1, were upregulated in response to *V. cholerae* 0139 (Table 2) compared to either control or *Lactobacillus* GG. IL-8, a potent chemoattractant of polymorphonuclear leucocytes, was the most highly upregulated (42.3-fold) in response to *V. cholerae* 0139. The IL-8 levels remained normal in *Lactobacillus* GG compared to medium alone. CXCL-1 (also known as growth related oncogene or GRO-α) showed a 14.5-fold increase. CXCL-10 (also known as interferon gamma-inducible protein or IP-10) showed a 16.07-fold increase in response to *V. cholerae* infection. CXCL-5 (also known as epithelial neutrophil activated peptide ENA78) was downregulated in response to *V. cholerae*.

The increase in IL-8 in response to *V. cholerae* was significantly attenuated by the simultaneous presence of *Lactobacillus* GG (−2.46-fold relative to *V. cholerae*, $P = 0.025$). *Lactobacillus* GG also decreased the CXCL-1 response to *Vibrio* (9.05 compared to 14.55, $P = 0.05$).

CC motif ligands

Members of this family of chemokines are in general chemoattractant to monocytes and macrophages. The monocyte chemoattractant protein (MCP) chemokines, CCL2 (MCP-1), CCL8 (MCP-2) and CCL 13 (MCP-4) were significantly downregulated in response to *V. cholerae* when compared to control and *Lactobacillus* GG (Table 2). *Lactobacillus* GG did not, by itself, significantly alter MCP chemokine levels in epithelial cells.

The macrophage inflammatory proteins (MIP), CCL 3 (MIP 1α) and CCL4 (MIP 1β) were also significantly downregulated in response to *V. cholerae* infection when compared to control and *Lactobacillus* (Table 2). The CCL5 chemokine (RANTES – regulated on activation normal t-cell expressed and secreted) was found to be not altered by either *V. cholerae* 0139 or *Lactobacillus* GG. Simultaneous presence of *Lactobacillus* GG did not alter significantly the changes in this family of chemokines induced by *V. cholerae* infection.

CC motif chemokine receptors

These function as receptors for the CC chemokines. CCR3, a receptor for the chemokines CCL 5, 7, 8 and 13, was downregulated in response to *V. cholerae* infection. CCR8, a receptor for the chemokines CCL and CCL 4, was significantly downregulated in response to *V. cholerae* infection when compared to control and *Lactobacillus* GG (Table 2).

CXC motif chemokine receptors

Expression of the chemokine CXC motif receptors was not altered in response to either *V. cholerae* 0139 or *Lactobacillus* GG or both in intestinal epithelial cells.

Other chemokines and related genes

Tumour necrosis factor alpha (TNF α), a proinflammatory gene, was increased significantly in response to *V. cholerae* when compared to control. Several other genes, including TREM 1, TCP 10, SLIT 2 and CMKLR1 were downregulated in response to *V. cholerae* infection. Presence of *Lactobacillus* GG did not alter the changes induced by *V. cholerae*. MMP-7, a member of the matrix metalloproteinase family involved in the breakdown of extracellular matrix, was significantly upregulated in response to *V. cholerae* infection when compared to control and *Lactobacillus* GG.

Modulation of the response to *Vibrio cholerae* of HT-29 cells by *Lactobacillus* GG

Lactobacillus GG in itself did not alter expression levels of any of the genes in this array. However, it modulated

the *V. cholerae* activation of CXCL8 and CXCL1, significantly reducing the increased expression of these genes in response to the pathogen.

Role of TLR4 in activation of IL-8 secretion in response to *Vibrio cholerae*

The efficiency of silencing was confirmed by quantitative RT-PCR using the TLR 4 specific primer with β-actin as a house keeping gene. shRNA targeting the TLR 4 gene, when transduced into HT-29 cells, demonstrated varying levels of knockdown. Three of the constructs (TRCN0000 0056893, TRCN0000056895 and TRCN0000056896) reduced the endogenous level of TLR 4 to less than 5% (3.6 ± 1.8%, 3.9 ± 1.3% and 4.9 ± 2.2% respectively, mean ± SEM), while one construct (TRCN0000056894) reduced it to 25% (25.8 ± 8.8%). Two of these (named shRNA3 and shRNA4 in our lab) which reduced TLR4 to less than 5% were infected with *V. cholerae* O139 and IL-8 secretion measured. TLR4 silencing was confirmed by protein blotting. There was no significant alteration in IL-8 secretion in response to *V. cholerae*, compared to normal HT29 cells exposed to this bacterium (Fig. 1). This suggests that TLR 4 is not involved in the pathway from recognition of *V. cholerae* by HT29 cells leading to IL-8 production.

NF-κB activation

IκBα degradation, indicative of NFκB activation, was noted in *V. cholerae* infected cells. The degradation of IκBα was time-dependent, occurring 30 min after contact with *V. cholerae* but returning to normal levels within 2 h after contact with the bacteria had been removed (Fig. 2). This degradation was prevented in cells that were exposed simultaneously to both *V. cholerae* and *Lactobacillus* GG (Fig. 3).

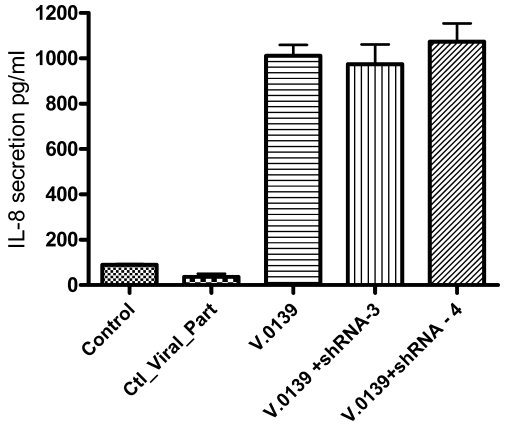

Figure 1 Interleukin-8 output from HT29 cells after exposure to *Vibrio cholerae*. Cells were untreated (no shRNA), or exposed to control lentiviral particles, or had TLR 4 silenced using appropriate shRNA (shRNA3 and shRNA4), confirmed by >95% reduction in mRNA levels of TLR 4. Values shown are mean (SEM) of five experiments.

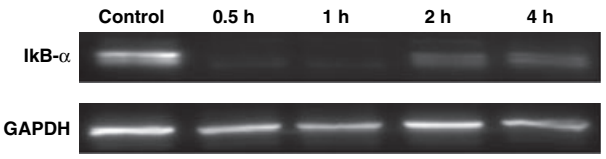

Figure 2 Activation of nuclear factor kappa B is shown in this Western blot. HT29 cells were infected with *Vibrio cholerae* O139 and cells taken at different time intervals, lysed and Western blot performed using antibody to IκBα and GAPDH. Loss of IκBα at the half and one time hour points after infection with faint bands reappearing at 2 and 4 h suggests activation of NFκB by the bacterium within half an hour after contact and diminishing within 2–4 h after contact with the bacterium is removed.

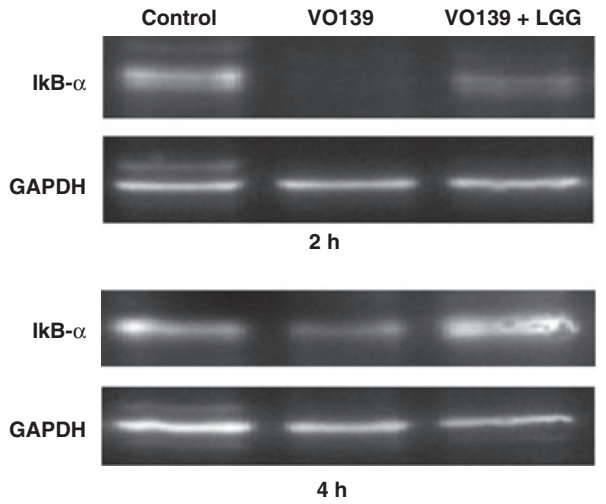

Figure 3 Activation of nuclear factor kappa B by *Vibrio cholerae* is prevented by the probiotic bacterium *Lactobacillus* GG. HT29 cells were infected with *V. cholerae* O139, lysed after 2 or 4 h and Western blot performed using antibody to IκBα and GAPDH. Loss of IκBα secondary to *V. cholerae* infection is not noted in HT29 cells that had been simultaneously exposed to both *V. cholerae* and *Lactobacillus* GG.

p38 MAP kinase pathway

Vibrio cholerae infected cells did not show phosphorylation of p38 MAP kinase at different time points of incubation (Fig. 4). This shows *V. cholerae*-induced changes in chemokine gene expression are unlikely to be through this pathway.

Discussion

These studies show that contact with *V. cholerae* regulated the expression of several chemokine and chemokine-related genes in the HT29 epithelial cell line. The pattern of activation is likely to provide information

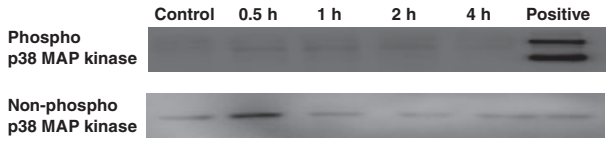

Figure 4 Western blot for phosphorylated and non-phosphorylated forms of p38 kinase in HT29 cells infected with *Vibrio cholerae* for varying time periods. The non-phosphorylated form is present in all cells, and there is no significant change in the phosphorylated form over time compared to control, suggesting that the pathogen did not activate this pathway.

regarding early events in pathogenesis resulting from the innate immune contact of the host with the pathogenic bacterium. In general, this interaction of the bacteria with epithelial cells resulted in several CXCL chemokines being upregulated while several CCL chemokines were downregulated. These effects enhance our understanding of the innate immune response induced by *V. cholerae*, but interpretation should be tempered by the fact that these studies were conducted using a human intestinal epithelial cell line and not using primary intestinal cells.

Members of the CXC family of chemokines, including IL-8, ENA78 and GRO-α, are neutrophil chemoattractants [8]. These were upregulated in response to *V. cholerae* infection. This family of chemokines is known to be important in the inflammatory and immune response elicited by some enteropathogens [11, 12]. Upregulation of TNF expression was noted, consistent with the general effect of *V. cholerae*. Upregulation of this gene group is expected to result in recruitment of polymorphonuclear leucocytes to the site of infection, and is likely responsible for the inflammatory manifestations associated with *V. cholerae* O139 infection, including the leucocyte infiltration and increase in inflammatory mediators noted in this infection.

Members of the CC chemokine family include RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted), monocyte chemoattractant proteins (MCP)-1, 2, 3 and 4, and the macrophage inflammatory proteins (MIP)-1α and ß, -3α and ß, the C chemokine family (e.g. lymphotactin), and the CX_3C chemokine family (e.g. neurotactin/fractalkine) primarily activate and recruit mononuclear cells such as monocytes/macrophages and lymphocytes which are mostly involved in T-helper type 1 (Th1) inflammation [8, 13]. These chemokines were downregulated in response to *V. cholerae* infection. These findings contrast with another recent study where *V. cholerae* induced upregulation of expression of MCP-1 in T84 cells but not in Caco2 cells [14]. It is possible that the specific pattern of chemokine regulation depends on the phenotype of the intestinal epithelial cell – the HT29 and Caco2 cells are absorptive villus-type cells, while the T84 cell has a secretory phenotype. An effective Th1 response resulting from innate signalling by the bacterium helps in the control of infection with *Mycobacterium tuberculosis* [15]. Although, the cellular defences are not as important in the control of cholera, it is possible that a downregulated Th1 response could be a potential survival mechanism for the bacterium. *V. cholerae* infection, however, did not activate the other members of this chemokine family, such as the eotaxins, that attract eosinophils, basophils and lymphocytes of the T-helper cell type 2 (Th2) phenotype. Cholera toxin is known to induce regulatory T cells and Th2 cells [16], which will also lead to downregulation of the Th1 response. This could suggest that recognition of *V. cholerae* by intestinal epithelial cells is likely to result in a downregulated Th1 response with a consequent tilt of the balance towards a Th2 predominance.

Of the related molecules that were activated, MMP-7 was upregulated in *V. cholerae* infection. MMP-7 is known to be induced in airway epithelial cells in *Pseudomonas aeruginosa* infection [17]. Matrix metalloproteinases are part of a family of neutral proteinases which are involved in the processing and activation of cytokines, chemokines and growth factors among a host of other actions, and may play a role in several gastrointestinal diseases including infectious gastroenteritis, inflammatory bowel disease and cancer [18, 19].

The signalling events that lead to IL-8 activation by *V. cholerae* were also examined. Previous studies have suggested that lipopolysaccharide and TLR-4 signalling may be important in the activation of pro-inflammatory pathways by *V. cholerae* [9, 20]. Mice with a mutation in the TLR4 gene showed attenuation of pro-inflammatory cytokine responses in response to *V. cholerae* [9]. Lipopolysaccharide derived from *V. cholerae* has been shown to be a potent activator of MyD88-dependent TLR4 signalling in macrophage cell lines [20]. The present study shows that TLR-4 was not involved in the pro-inflammatory signalling of *V. cholerae* O139 to the HT29 epithelial cell line as silencing the TLR-4 gene did not diminish the IL-8 response. It is possible that this anomalous finding is related to the fact that we examined only a single intestinal epithelial cell line, and that the signalling mechanism of the bacterium to different cells varies. In keeping with others, we found that nuclear factor kappa B activation accompanies the effect of *V. cholerae* on epithelial cells.

In the present study, we did not examine the effect of cholera toxin on pro-inflammatory gene activation in the intestinal epithelial cell line. Cholera toxin is a potent vaccine adjuvant when given by the mucosal route. It appears to modulate the expression of costimulatory molecules on dendritic cells and inhibit the production of chemokines and cytokines [16]. However, other investigators have shown that toxin-deficient strains of *V. cholerae* can induce expression of pro-inflammatory cytokines and chemokines [14].

In conclusion, it is likely that *V. cholerae* on contact with the intestinal epithelium induces the secretion of

chemokines that attract neutrophils and inhibit the secretion of chemokines that can attract monocytes and macrophages. As the innate response can modify the adaptive immune response, it is likely from these findings that this organism through its epithelial cell interaction induces a Th2 type response. The inhibition of the neutrophil chemokine response to *V. cholerae* by *Lactobacillus* GG suggests that the anti-inflammatory effect of the probiotic bacterium is not confined to inflammatory bowel disease and may extend to interactions of the epithelium with infective organisms as well.

Acknowledgment

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