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Differential Interleukin-8 Response of Intestinal Epithelial Cell Line to Reactogenic and Nonreactogenic Candidate Vaccine Strains of *Vibrio cholerae*

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In this study, we analyzed whether attachment of *Vibrio cholerae* vaccine strains to human intestinal epithelial cells can induce an interleukin-8 (IL-8) response. The IL-8 transcripts were detected by PCR amplification of reverse-transcribed mRNA, and the gene product secretion was measured by an enzyme-linked immunosorbent assay. Infection of monolayers of the undifferentiated HT29-18N2 cell line with reactogenic (JBK70 and 81) and nonreactogenic (CVD103HgR and 638) vaccine strains of *V. cholerae* resulted in markedly higher IL-8 expression by epithelial cells exposed to reactogenic strains than by cells exposed to the nonreactogenic strains. Additionally, epithelial cells produced IL-8 transcripts following stimulation with cholera vaccine strains in a concentration-dependent manner. These results represent a new insight into the inflammatory component of reactogenicity and could be used as a predictive marker of vaccine reactogenicity prior to human testing.

Cholera is traditionally considered to be a noninflammatory diarrheal disease caused by *Vibrio cholerae* serogroups O1 and O139 (10). Several groups of investigators have constructed a wide range of live attenuated *V. cholerae* O1 and O139 strains by deleting the CTXϕ genes in order to immunize against cholera (2, 5, 9, 11, 16, 19). However, many of these strains have shown residual adverse properties (reactogenicity) in volunteer studies (1, 12, 22, 23). The cellular basis of reactogenicity is not clear, but there is some evidence of an intestinal inflammatory response. It was postulated that close proximity or contact of bacterial cells to the apical surface of the intestinal epithelium causes reactogenicity, due to the induction of a local inflammatory response; this theory was based on the lower reactogenicity observed for nonmotile mutants of *V. cholerae* compared with parental motile vaccine strains in volunteer studies (15). Furthermore, oral vaccination with CVD110, a reactogenic strain, produced copious amounts of lactoferrin and increased interleukin-8 (IL-8) levels in the stool of volunteers, while a nonreactogenic strain (CVD103HgR) did not (21). The proinflammatory cytokine IL-8 is a potent chemoattractant for polymorphonuclear leukocytes and T lymphocytes (17). Polymorphonuclear leukocytes, which are found in large numbers in inflammatory diarrheas (6, 20), can induce chloride secretion similar to that seen in toxigenic secretory diarrhea illnesses (14) and could play an important role in the diarrhea seen with live attenuated cholera vaccine strains. On the other hand, epithelial cells infected with several invasive and some noninvasive enteric pathogens produced an IL-8 response (8). However, the induction of proinflammatory signals by the attachment of *V. cholerae* strains to intestinal epithelial cell lines has not been investigated. Here, we examined the impact of attachment of reactogenic and nonreactogenic live cholera vaccine strains to human intestinal epithelial cells on the IL-8 response by using the undifferentiated HT29-18N2 cell line (7), a clone derived from the HT29 human adenocarcinoma cell line. This clone, when maintained in high-glucose Dulbecco's modified medium supplemented with fetal serum, does not produce mucus and mainly remains in the form of columnar enterocytes (18).

Strains, cell cultures, and infection protocol. The *V. cholerae* strains used in this work are summarized in Table 1 and were grown as semiconfluent streaks on Luria-Bertani agar. The HT29-18N2 cell line was provided by Tom E. Phillips (Department of Molecular Microbiology and Immunology, University of Missouri, Columbia) and was maintained in a high-glucose Dulbecco's modified medium supplemented with 10% fetal calf serum, penicillin-streptomycin (10 U and 10 μg per ml), and amphotericin B (0.5 μg/ml), hereafter referred to as complete medium. The cells were incubated at 37°C in complete medium in an atmosphere of 5% CO_2. For all experiments, undifferentiated HT29-18N2 cells were used to avoid mucus interference with adherence for all strains assayed. Attachment of *V. cholerae* vaccine strains to the intestinal epithelial cell line was essentially determined as described previously (3). For IL-8 mRNA and gene product detection, HT29-18N2 cells were seeded at 2×10^4 to 3×10^4 cells/cm^2 onto 25-mm-diameter coverglasses in six-well tissue culture plates in complete medium and incubated at 37°C in 5% CO_2 until confluent. Twenty-four hours before stimulation, the cell cultures were washed and maintained in fresh complete medium without fetal calf serum. Duplicate coverglasses were exposed to 10^6 to 10^7 CFU of each cholera vaccine strain for 30 min, washed three times, transferred to fresh Dulbecco's medium,

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TABLE 1. Adherence of different *V. cholerae* vaccine strains to the undifferentiated HT29-18N2 colonic epithelial cell line

Strain	Relevant genotype and/or phenotype	Log CFU/well[a]				
		Inoculum[b]	Adherence after[c]:			
			0.5 h	2 h	4 h	8 h
81	Δ(*ctxA ctxB zot ace orfU cep*), O1, El Tor, Ogawa mutant from C7258	6.94 ± 0.29	6.43 ± 0.15	7.81 ± 0.28	9.00 ± 0.39	8.90 ± 0.23
638	*hap*::*celA* mutant from 81	7.27 ± 0.14	6.41 ± 0.08	8.00 ± 0.42	8.85 ± 0.35	9.34 ± 0.28
JBK70	Δ(*ctxA ctxB*), O1, El Tor, Inaba mutant from N16961	7.02 ± 0.44	6.88 ± 0.34	8.34 ± 0.22	9.36 ± 0.40	8.08 ± 0.27
CVD103HgR	Δ(*ctxA*), *hlyA*::*mer*, O1, Classical, Inaba mutant from 569B	7.67 ± 0.081	6.84 ± 0.20	8.74 ± 0.35	9.39 ± 0.33	8.53 ± 0.22

[a] Mean log CFU/well from three independent experiments ± SEM.
[b] Inoculum size of different *V. cholerae* strains to undifferentiated HT29-18N2 cells in three independent experiments.
[c] The number of vibrios attached to HT29-18N2 on two replicate coverglasses from three independent experiments. No significant ($P < 0.05$) differences were observed among the cholera vaccine strains tested, at all times assayed.

and incubated for up to 8 h. In dose-dependency experiments, the *V. cholerae* inoculum size ranged from 10^4 to 10^7 CFU/well and RNA extractions were performed 4 h postinfection.

RNA extraction, RT-PCR analysis, and cytokine assay. Total cellular RNA was extracted 0.5, 2, 4, and 8 h after challenge of the epithelial cells with the different *V. cholerae* vaccine strains by using the acid guanidinium thiocyanate-phenol-chloroform method (4). First-strand cDNA synthesis was performed by using 1.5 μg of each purified RNA primed with 2 pmol of oligo(dT) primer in a 20-μl reaction volume. Reverse transcriptions (RTs) were made by using the Reverse Transcription system (Promega, Madison, Wis.). Qualitative PCR analysis was performed with 1 μl of each cDNA in a 50-μl reaction volume containing 0.2 mM deoxynucleoside triphosphate, 2 mM Mg^{2+}, 0.5 μM concentrations of each sense and antisense primer, and 1.25 U of *Taq* DNA polymerase. All reagents for PCR amplification were purchased from Boehringer (Mannheim, Germany). The PCR profile included a 94°C denaturalization for 5 min, followed by 30 cycles of denaturalization at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The primers used and the molecular sizes of the corresponding products were as follows: β-actin sense (5′ GTG GGG CGC CCC AGG CAC CA 3′) and antisense (5′ CTC CTT AAT GTC ACG CAC GAT TTC 3′) (548 bp); IL-8 sense (5′ ATG ACT TCC AAG CTG GCC GTG 3′) and antisense (5′ TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC 3′) (302 bp). The IL-8 concentration was determined at 0.5, 2, 4, and 8 h postinfection in culture supernatants of undifferentiated HT29-18N2 cells stimulated with reactogenic and nonreactogenic cholera vaccine strains by the Quantikine enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, Minn.), with a detection limit of less than 10 pg/ml. Both the RT-PCR analysis and cytokine assay used unstimulated epithelial cell cultures as controls. Tumor necrosis factor alpha (TNF-α)-treated epithelial cells were used as positive controls for IL-8 stimulation.

Adherence of *V. cholerae* vaccine strains to undifferentiated HT29-18N2 cells was evaluated at 0.5, 2, 4, and 8 h postinfection, and no significant ($P < 0.05$) differences were observed among the cholera vaccine strains tested at all times assayed (Table 1). Epithelial cell viability was higher than 95% within 8 h, but longer times were not analyzed due to drastic changes in cell morphology and low cell viability (less than 80%). The time course of IL-8 mRNA expression in epithelial cells after exposure to cholera vaccine strains was examined. In unstimulated cells no IL-8 transcripts were detected, while in TNF-α-treated cells a strong IL-8 mRNA expression was seen in 4 h (data not shown). Amplification of the same cDNAs with primers for β-actin demonstrated that the expression of the transcripts for this constitutive protein was unaffected in all samples tested. The IL-8 mRNA induction by strain JBK70 became evident at 2 h and increased for up to 8 h (Fig. 1); similar results were observed for strain 81, except that IL-8 mRNA was evident 1.5 h earlier (Fig. 1). On the other hand, the nonreactogenic strains CVD103HgR and 638 also induced IL-8 mRNA within 2 h which also increased for up to 8 h (Fig. 1). Strikingly, IL-8 mRNA expression in the nonreactogenic strains was, at all sampling times, markedly lower than that induced by strain JBK70 or 81. Increases in IL-8 mRNA were dependent on inoculum size, as demonstrated in dose-dependency experiments for both reactogenic and nonreactogenic strains, with lower levels of transcript expression in the latter

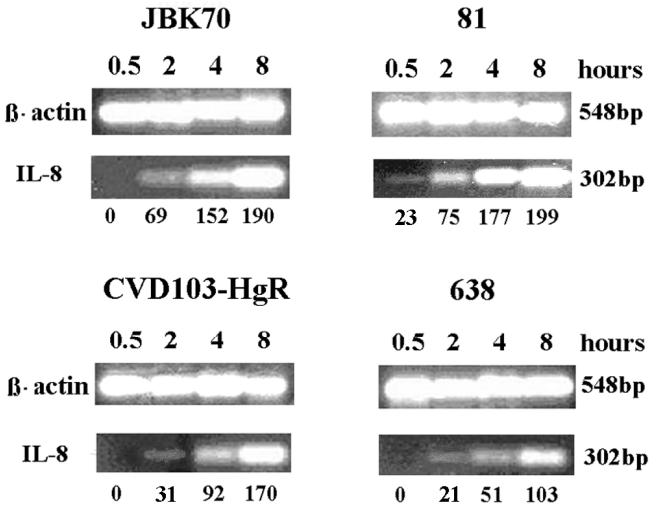

FIG. 1. Time course of IL-8 mRNA induction in undifferentiated HT29-18N2 colonic epithelial cells by reactogenic and nonreactogenic cholera vaccine strains. Data shown are from a representative gel electrophoresis of three independent RT-PCR amplification products of β-actin and IL-8 mRNAs from undifferentiated HT29-18N2 epithelial cells, after stimulation with four *V. cholerae* vaccine strains at various time intervals from 0 to 8 h. Numbers under each panel represent densitometry values and are expressed in arbitrary units. Sizes are indicated in base pairs (bp).

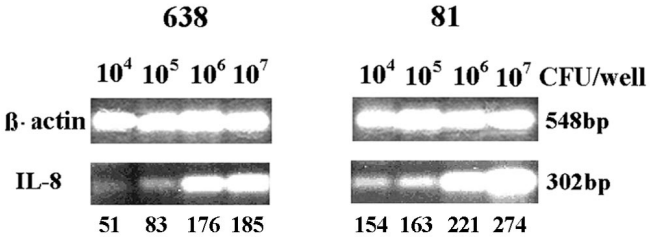

FIG. 2. Dose-dependency experiments for IL-8 mRNA induction in undifferentiated HT29-18N2 colonic epithelial cells by reactogenic and nonreactogenic cholera vaccine strains. Data shown are from a representative gel electrophoresis of three independent RT-PCR amplification products of β-actin and IL-8 mRNAs from undifferentiated HT29-18N2 epithelial cells, after 4 h of stimulation with two *V. cholerae* vaccine strains; inoculum size ranged from 10^4 to 10^7 CFU/well. Numbers under each panel represent densitometry values and are expressed as arbitrary units. Sizes are indicated in base pairs (bp).

(Fig. 2). Supernatants were collected from *V. cholerae*-infected confluent monolayers and IL-8 secretion was measured by enzyme-linked immunosorbent assay. Unstimulated cells did not produce detectable levels of IL-8 (i.e., levels were below 10 pg/ml), while TNF-α induced potent secretion of the IL-8 gene product within 4 h (Table 2). Both reactogenic strains (JBK70 and 81) induced secretion of IL-8, starting at 4 h (Table 2). In contrast, no IL-8 was detected when epithelial cells were exposed to 638, while the IL-8 gene product was only detectable for CVD103HgR at 8 h (Table 2).

Chemokines belonging to the C-X-C intercrine family of cytokines, such as IL-8, play a major role in mobilizing cellular defense mechanisms to eliminate pathogens by recruiting and activating neutrophils and T cells (17). Neutrophils are a protagonist cell type involved during the earlier steps of inflammatory reactions, with a capacity to interact with both epithelial and endothelial cells as well as an ability to produce a wide range of mediators. As IL-8 and other proinflammatory cytokines secreted by epithelial cells may be the initial signals for an acute inflammatory response following bacterial invasion of mucosal surfaces (8), we sought to develop an in vitro model for IL-8 induction in intestinal epithelial cells, challenged with different *V. cholerae* vaccine strains, to characterize this host-vaccine interaction. Our results demonstrated that JBK70, a reactogenic strain used in North American and Cuban volunteer studies (1, 12) and 81, a highly reactogenic strain used in Cuban volunteers (unpublished data), induced a markedly higher IL-8 response in terms of both mRNA and protein levels in the undifferentiated HT29-18N2 cell line than the response produced by the nonreactogenic strains, CVD103HgR and 638 (1, 13). The differential IL-8 response obtained here for reactogenic and nonreactogenic strains is in agreement with the reactogenic hypothesis of Mekalanos et al. (15) and with the recent report of Silva et al. (21) in which the stools of volunteers colonized with CVD110 contained increased levels of this cytokine. Taken together, these findings support the idea that IL-8 may be an important mediator in the proposed inflammatory response to reactogenic cholera vaccine strains, suggesting a potential role for neutrophils in the diarrhea seen with live attenuated *V. cholerae* vaccine strains in clinical trials. Further studies using other reactogenic and nonreactogenic *V. cholerae* vaccine strains will be needed to evaluate whether the IL-8 response seen in HT29-18N2 cells could be used as a predictive marker of vaccine reactogenicity prior to human testing. Experiments are being conducted to determine the expression of other proinflammatory and anti-inflammatory cytokines by this intestinal epithelial cell line in response to the same stimuli.

TABLE 2. Secretion of IL-8 by the undifferentiated HT29-18N2 cell line exposed to reactogenic or nonreactogenic cholera vaccine strains

Strain or treatment	Mean IL-8 concn (pg/ml) ± SEM[a]	
	4 h (n = 3)	8 h (n = 3)
81	32.4 ± 0.64	1,396 ± 13
638	<10	<10
JBK70	32.3 ± 0.6	486 ± 3.4
CVD103HgR	<10	32.4 ± 0.52
TNF-α[b]	13,775 ± 250.1	ND

[a] Background secretion of IL-8 in unstimulated HT29-18N2 cultures at 8 h was <10 pg/ml. ND, not determined.
[b] TNF-α-treated cells were included as a positive control.

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