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Vaccine strains of *Vibrio cholerae* induce a differential array of proinflammatory mediators in an intestinal epithelial cell line

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Key words: *Vibrio cholerae*, vaccine strains, reactogenicity and inflammation.

RESUMEN. En este estudio se evaluó la expresión de varios mediadores inflamatorios en la línea celular HT29-18N2 en respuesta a la interacción con cepas vacunales reactogénicas y no reactogénicas de *Vibrio cholerae*. Los transcriptos de todos los mediadores fueron detectados por PCR al amplificar los ADN de simple cadena obtenidos en las reacciones de transcripción reversa, mientras que la secreción de los mediadores inflamatorios fue medida con un inmunoensayo de enzima ligada. Las incubaciones conjuntas de la línea celular de epitelio intestinal HT29-18N2 con las cepas vacunales reactogénicas (JBK70 y 81) y no reactogénicas (CVD103HgR y 638), indujo la expresión diferencial de tres mediadores inflamatorios (IL-8, GM-CSF y TNFα) en la línea celular, esta expresión fue siempre mayor en las células epiteliales expuestas a las cepas vacunales reactogénicas. Adicionalmente, las células muertas por calor de las cepas vacunales 81 y 638 no indujeron la producción de transcriptos del TNF-α, pero sí los de IL-8 y GM-CSF, así como también, activaron la producción de la IL-8 en la línea HT29-18N2; sin embargo, en todos los casos, los niveles de expresión fueron inferiores a los inducidos por las cepas vacunales vivas. Por el contrario, no se detectó la expresión de ninguno de los mediadores inflamatorios estudiados al usar adaptadores Tranwell que impedían la interacción directa entre las cepas vacunales vivas y las células epiteliales. Además, ninguna de las cepas vacunales de *Vibrio cholerae* empleadas fueron capaces de inducir la producción de los ARNm de IL-1α, MCP-1, TGF-β1 o INOS. Estos resultados demuestran que las cepas vacunales de cólera inducen una expresión diferencial de mediadores inflamatorios en células epiteliales y que esta inducción es dependiente del contacto estrecho de los vibriones con dichas células. Lo anterior sugiere que estas interacciones cercanas pudieran jugar un papel importante en la reactogenicidad, mediada por una respuesta inflamatoria del hospedero, de las vacunas vivas de cólera.

ABSTRACT. In this study, the expression of inflammatory mediators by the HT29-18N2 cell line in response to reactogenic and nonreactogenic *Vibrio cholerae* live vaccine strains was evaluated. All transcripts were detected by PCR amplification of reverse-transcribed mRNA, and the secretion of the inflammatory mediators were measured by enzyme-linked immunosorbent assays. Coincubations of monolayers of the HT29-18N2 cell line with reactogenic (JBK70 and 81) and nonreactogenic (CVD103HgR and 638) cholera vaccine strains resulted in the expression of a differential array of three proinflammatory mediators (IL-8, GM-CSF and TNFα), which was always higher in epithelial cells exposed to reactogenic vaccine strains. Additionally, heat-killed cells from 81 and 638 strains did not induce epithelial cells to produce TNF-α transcripts, while IL-8 and GM-CSF mRNAs and the IL-8 protein were induced, albeit at lower levels than live strains. In contrast, none of the mediators studied were detected either at mRNA or protein level when Transwell devices were used to interfere live-vaccine/HT29-18N2 interactions. Notably, mRNAs for IL-1α, MCP-1, TGF-β1 or INOS were not induced by *Vibrio cholerae* strains. These results demonstrate that cholera vaccine strains induce a differential proinflammatory cytokine expression in which direct contact of vibrios with intestinal cells is needed, suggesting that close interactions could play a central role in the live cholera vaccine reactogenicity derived from a host inflammatory response.

INTRODUCTION

Cholera is a watery diarrheal disease caused by *Vibrio cholerae* of serogroups O1 and O139.[1] Many efforts have been done to obtain a cholera vaccine but a fully effective vaccine is still to be obtained. Live cholera vaccines seem to be one of the most promising strategy to reach this objective, accordingly, several live attenuated *V. cholerae* O1 and O139 strains have been constructed by deleting the CTXΦ and other virulence associated genes to immunize against cholera.[2-7] However, there are two main problems when

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live vaccines are used: first, live bacteria are excreted to the environment with the concomitant risk of reacquisition and dissemination of cholera toxin genes[8,9] and second, most live vaccine candidates have shown untoward reactions (reactogenicity) in volunteer studies.[10-13] The molecular basis of reactogenicity is not well understood, although a hypothesis of an intestinal inflammatory reaction has been postulated based on volunteer studies of nonmotile cholera vaccine strains.[14] For the first time, the inflammatory hypothesis was evidenced when an oral vaccination with CVD110, a reactogenic strain, produced copious amounts of lactoferrin and increased interleukin-8 levels in the stools of volunteers, while the nonreactogenic strain CVD103HgR did not.[15] New evidences into the inflammatory component of reactogenicity came from a previous report, in which the IL-8 induction in undifferentiated HT29-18N2 cells exposed to live cholera vaccines was markedly higher for reactogenic strains than for nonreactogenic ones.[16] Also it has been suggested that reactogenicity can be caused by some X-reactogenic factor(s). In a recent study, measuring the electrical response of T84 intestinal epithelial cell line to culture supernatants from various vaccine strains, it was found that the Hemagglutinin-protease (HA-protease) of *V. cholerae* causes a loss of transepithelial resistance (TER) across a T84 monolayer.[17] This result suggests that HA-protease and possibly other virulence factors could induce reactogenicity by a noninflammatory mechanism. On the other hand, epithelial cells are increasingly being recognized to participate in inflammatory and immune reactions. In fact, intestinal epithelial cells express proinflammatory signals in response to bacterial injury.[18-20] However, the intestinal epithelial cytokines response to *V. cholerae* has not been studied yet and only the IL-8 induction in undifferentiated HT29-18N2 cells by live cholera vaccine strains has been published.[16] Here, it was examined the expression of a wide range of inflammatory mediators by the HT29-18N2 cell line exposed to reactogenic and nonreactogenic live cholera vaccine strains, as well as to heat-killed vaccine strains. The contribution of vibrios-intestinal cell interactions in the epithelial cytokines response was also analyzed.

MATERIALS AND METHODS

Strains, cell cultures and infection protocol

All *Vibrio cholerae* strains used in this work (Table 1) were grown as semiconfluent streaks on Luria-Bertani agar (triptone, 1 %, yeast extract 0.5 %, sodium chloride 1 %, technical agar 1 %). The HT29-18N2 cell line was maintained in a high glucose Dulbecco's modified medium (GIBCOBRL, Cat. number 52100-047) supplemented with 10 % fetal calf serum (FCS), penicillin-streptomycin (10 U/mL and 10 μg/mL) and fungizone (0.5 μg/mL), hereafter referred 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[21,22] and the attachment of *V. cholerae* vaccine strains to the epithelial cell line was essentially determined as was described previously.[23] For inflammatory mediator mRNAs and gene products detection, HT29-18N2 cells were seeded at $2 \cdot 10^4$ to $3 \cdot 10^4$ cells/cm^2 on to 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 Dulbecco's medium without fetal calf serum or antibiotic. Duplicated wells were exposed to 10^7 Colony Forming Units (CFU) of each live cholera vaccine strain for 30 min, washed three times, filled with fresh Dulbecco's medium, and incubated for up to eight hours. In Transwell experiments, commercially available 24-mm and 0.1 μm Transwell devices (Costar Laboratories, Cambridge, Mass.) were inserted into six well-confluent-monolayers of HT29 18N2 cells and filled with 1 mL of Dulbecco's medium containing 10^7 CFU of reactogenic cholera vaccine strains. Duplicated Transwell inserts were incubated in 5 % of CO_2 at 37 °C during 8 h. For heat-killed strains, HT29-18N2 cells were exposed to 10^8 vibrios/well during 8 h . Cholera vaccine strains had been killed by a heat treatment, which maintained cellular integrity and preserved relevant surface antigens.[24]

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 cell line with the different live or heat-killed *V. cholerae* vaccine strains by using the acid guanidinium thiocyanate-phenol-chloroform method.[25] Following the same procedure, RNA was obtained from HT29-18N2 cells exposed to reactogenic live strains grown in Transwell inserts. RT-PCR analysis were performed as described previously[16] by using 1.5 μg of each purified RNA for reverse transcriptions and 1 μL of each generated cDNA, with different sets of primers (Table 2), for PCR. The IL-8, GM-CSF and TNF-α protein concentrations

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

Strains	Relevant genotype and/or phenotype[Reference]	Log CFU/well[a]	
		Inoculum[b]	Adherence after[c]: 30 min
81	ΔCTXΦ prophage, O1, El Tor, Ogawa mutant from C7258[2]	7.27 ± 0.21	7.13 ± 0.13
638	*hap::celA* mutant from 81[7]	7.33 ± 0.17	7.25 ± 0.20
JBK70	Δ(*ctxA ctxB*), O1, El Tor, Inaba mutant from N16961[11]	7.18 ± 0.11	7.10 ± 0.13
CVD103-HgR	Δ(*ctxA*), *hlyA::mer*, O1, Classical, Inaba mutant from 569B[11]	7.17 ± 0.16	7.07 ± 0.16

[a] The mean of Log CFU/well from three independent experiments ± SD. [b] Inoculum size of different *V. cholerae* strains to undifferentiated HT29-18N2 cells in three independent experiments. No significant ($P > 0.05$) differences were observed on the inoculum size of different strains, ANOVA test. [c] The number of vibrios attached to HT29-18N2 on two replicate wells from three independent experiments. No significant. ($P > 0.05$) differences in adherence were observed among the cholera vaccine strains tested, ANOVA test.

Tabla 2. Primers used and the corresponding PCR products sizes.

Primers	Sequencea	PCR product sizes (bp)
β-actin	5' GTG GGG CGC CCC AGG CAC CA 3'	548
	5' CTC CTT AAT GTC ACG CAC GAT TTC 3'	
IL-1-α	5' GTC TCT GAA TCA GAA ATC CTT CTA TC 3'	420
	5' CAT GTC AAA TTT CAC TGC TTC ATC C 3'	
IL-8	5' ATG ACT TCC AAG CTG GCC GTG 3'	302
	5' TTA TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC 3'	
INOS	5' AAG CCC CAA GAC CCA GTG CC 3'	237
	5' CCA GCA TCT CCT CCT GGT AGA T 3'	
TGF-β1	5' AAC ATG ATC GTG CGC TCT GCA AGT GCA GC 3'	200
	5' AAG GAA TAG TGC AGA CAG GCA GGA 3'	
TNF-α	5' ATG AGC ACT GAA AGC ATG ATC 3'	702
	5' TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG CCC 3'	
GM-CSF	5' ACA CTG CTG AGA TGA ATG AAA CAG TAG 3'	286
	5' TGG ACT GGC TCC CAG CAG TCA AAG GGG ATG 3'	
MCP-1	5' TCT GTG CCT GCT GCT CAT AGC 3'	510
	5' GGG TAG AAC TGT GGT TCA AGA GG 3'	

[a] For each primer pair, the sense primer is given above the antisense primer.

were determined, in all experiments, at 8 h postinfection in culture supernatants of undifferentiated HT29-18N2 cells by Quantikine enzyme-linked immunosorbent assays (R&D System, Minneapolis, Minn.), with detection limits of less than 10, 3 and 4.4 pg/mL, respectively. Both, RT-PCR analysis and the immunoassays used unstimulated epithelial cell cultures as controls. An induction mixture (TNF-α, 100 ng/mL; IL-1α, 10 ng/mL and IFN-γ, 100 U/mL) was used to stimulate the HT29-18N2 cell line to produce a repertoire of inflammatory mediators.

RESULTS

Adherence of live cholera vaccine strains to HT29-18N2 cell line was evaluated at 30 min after infection and no significant differences ($P > 0.05$) were observed among cholera vaccine strains tested (Table 1). The epithelial cell viability was higher than 95% and attachment of these vaccine strains to the epithelial cell line was not different between them (data not shown) during the 8 h of the experiments as previously reported.[16]

The time course of inflammatory mediators mRNA expression in epithelial cells after challenge with cholera vaccine strains was examined. In unstimulated HT29-18N2 cells no transcripts were detected for any of the inflammatory mediators assayed, while in cells exposed to the induction mixture, a time-dependent expression of five inflammatory mediators was observed, including transcripts for IL-8, TNF-α, GM-CSF, INOS and MCP-1, (Fig. 1). In all cases, 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 (Figures 1, 2, 3 and 4). Infection of HT29-18N2 cells with reactogenic and nonreactogenic live cholera vaccine strains, resulted in the coordinate expression of a differential array of three proinflammatory mediators, IL-8, GM-CSF and TNFα as assessed by mRNA levels (Fig. 2). The IL-8 and GM-CSF mRNA induction by strains JBK70 and 81 became evident at 30 min and increased up to 8 h (Fig. 2). On the other hand, the nonreactogenic strains CVD103-HgR and 638 induced the IL-8 and GM-CSF transcripts 1.5 h later than reactogenic strains (Fig. 2). Being of note, IL-8 and GM-CSF mRNA expression in the nonreactogenic strains were, at all sampling times, at least 1.5 fold lower than those induced by strains JBK70 or 81 (Fig. 2). Additionally,

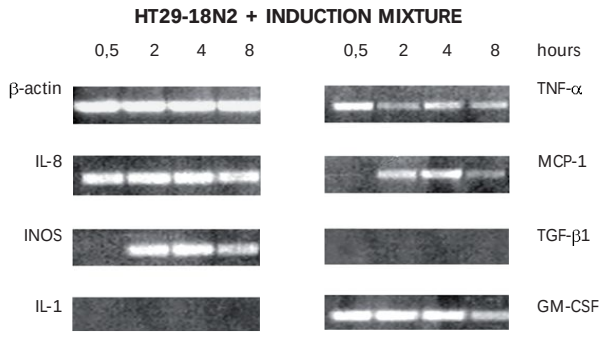

Fig. 1. *Time course induction of inflammatory mediators mRNA in undifferentiated HT29-18N2 colonic epithelial cells by a specific induction mixture. Data shown are from a representative gel electrophoresis of three independent RT-PCR amplification products of β-Actin, IL-1α, IL-8, INOS, TGF-β1, MCP-1, TNF-α and GM-CSF mRNAs from HT29-18N2 cells, after a direct stimulation with an induction mixture (TNF-α, 100 ng/mL; IL-1α, 10 ng/mL and IFN-γ,100 U/mL) at the time intervals shown.*

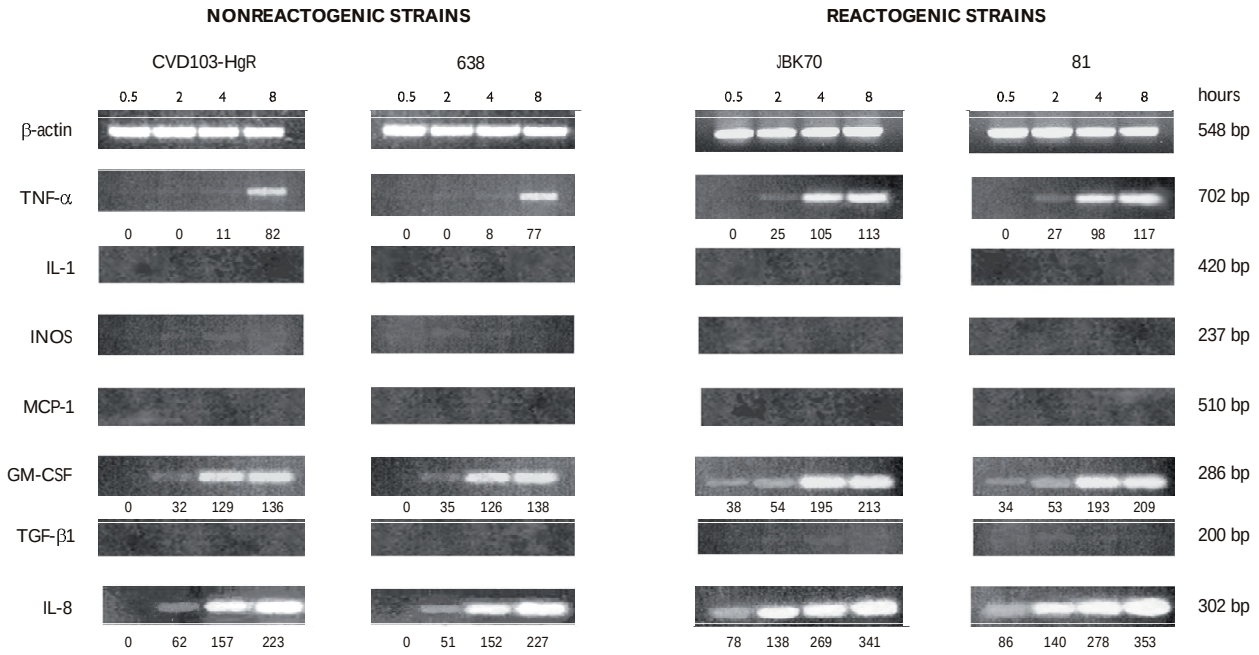

Fig. 2. *Time course induction of inflammatory mediators mRNA in undifferentiated HT29-18N2 colonic epithelial cells by reactogenic and non-reactogenic cholera vaccine strains. Data shown are from a representative gel electrophoresis of three independent RT-PCR amplification products of β-Actin, IL-1α, IL-8, INOS, TGF-β1, MCP-1, TNF-α and GM-CSF mRNAs from HT29-18N2 cells, at various time intervals, after stimulation with four V. cholerae vaccine strains. Numbers under panels represent densitometry values and are expressed in arbitrary units. Sizes are indicated in base pairs (bp).*

the reactogenic strains induced the intestinal epithelial cells to express TNF-α mRNA at two hours post infection that increased up to 8 h (Fig. 2), while for nonreactogenic strains the mRNA expression was only fully evident at 8 h (Fig. 2). Furthermore, heat-killed strains, 81 and 638, did not induce epithelial cells to produce TNF-α transcripts, while IL-8 and GM-CSF mRNA were produced, but at markedly lower levels than for live vaccines (Fig. 3). In contrast, the IL-8, TNF-α and GM-CSF genes expression was not observed in Transwell experiments with reactogenic live vaccine strains, even when the induction mixture (positive inductor) added inside transwell devices was able to induce transcripts for all three inflammatory mediators (Fig. 4). Notably, the HT29-18N2 cell line did not express mRNA for MCP-1 or INOS in response to the cholera vaccine strains tested (Fig. 2), while epithelial cells IL-1α and TGF-β1 transcripts were not produced in response to any of the stimulus (Figures 1 and 2).

Supernatants were collected from *V .cholerae*-infected confluent monolayers at 8 h (due to the higher mRNA expression of all inflammatory mediators at this time) to measure the secretion of these mediators by enzyme-linked immunosorbent assays. Unstimulated cells did not produce detectable levels of IL-8, TNF-α and GM-CSF, while the mixture (TNF-α, IL-1α and IFN-γ) induced the IL-8 epithelial gene product secretion (Table 3). Reactogenic and nonreactogenic vaccine strains induced IL-8 secretion, which was at least 1.4 fold higher in epithelial cells challenged with reactogenic strains, while TNF-α secretion was only detected in epithelial cells exposed to reactogenic strains (Table 3). Additionally, heat-killed vaccine strains induced HT29-18N2 cells to secrete IL-8, but not TNF-α, at levels of at least 3 and 1.7 fold lower than live reactogenic and nonreactogenic strains, respectively (Table 3). Supernatants were also tested for GM-CSF secretion; however, in spite of the fact that by PCR the intestinal epithelial cell line expressed mRNA for this inflammatory mediator (Figures 1, 2 and 3), GM-CSF was not secreted in measurable amounts by epithelial cells in response to *V. cholerae*, but was produced (1 526 ± 154.2 pg/mL) by epithelial cells exposed to the induction mixture, although at unexpected low levels if compared with the mRNA levels and the protein concentration obtained for IL-8 (Fig. 1) and (Table 3).

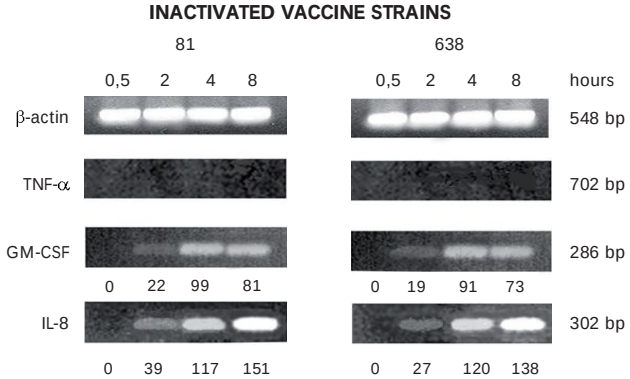

Fig. 3. *Time course induction of inflammatory mediators mRNA in undifferentiated HT29-18N2 colonic epithelial cells by heat inactivated cholera vaccine strains. Data shown are from a representative gel electrophoresis of three independent RT-PCR amplification products of β-Actin, IL-8, TNF-α and GM-CSF mRNAs from HT29-18N2 cells, at various time intervals, after stimulation with two heat-killed V. cholerae vaccine strains. Numbers under panels represent densitometry values and are expressed in arbitrary units. Sizes are indicated in base pairs (bp).*

Tabla 3. Secretion of IL-8 and TNF-α by the undifferentiated HT29-18N2 cell line exposed to live (reactogenic and nonreactogenic) or heat inactivated cholera vaccine strains.

Strain or treatment	Mean cytokines conc. (pg/ml) ± SD[1]	
	IL-8[2] (n = 6)	TNF-α[2] (n = 6)
81	9 870 ± 1 200[a]	107 ± 11
638	5 190 ± 710[b]	< 4.4
JBK70	16 440 ± 1 800[c]	142 ± 18
CVD103-HgR	5 630 ± 740[b]	< 4.4
81[3]	2 480 ± 340[d]	< 4.4
638[3]	1 670 ± 240[e]	< 4.4
Induction mixture[4]	43 700 ± 3 450	NT

[1] Mean of IL-8 and TNF-α concentrations ± SD measured at 8 h postinfection on two replicate wells from three independent experiments. [2] Background secretions of IL-8 and TNF-α in unstimulated HT29-18N2 cultures at 8 h were < 10 and 4.4 pg/mL, respectively. [3] Heat-killed vaccine strains. [4] A mixture (TNF-α, 100 ng/mL; IL-1α, 10 ng/mL and IFN-γ,100 U/mL) was used as a positive inducer of epithelial cells. NT: No tested due to TNF-α was exogenous added with the induction mixture. [a, b, c, d, e] Different letters indicates significant differences ($p < 0.05$), multiple ranges Duncan test.

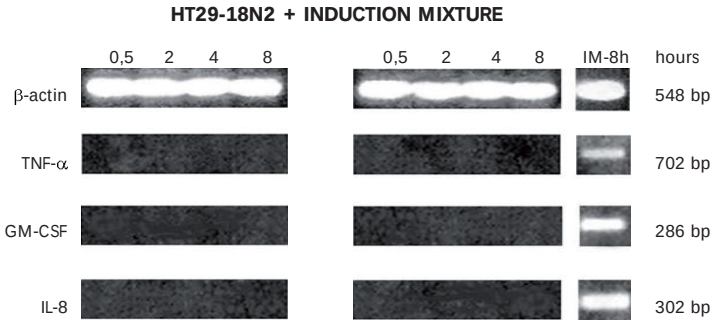

Fig. 4. *Time course induction of inflammatory mediators mRNA in undifferentiated HT29-18N2 colonic epithelial cells. Data shown are from a representative gel electrophoresis of three independent RT-PCR amplification products of β-Actin, IL-8, TNF-α and GM-CSF mRNAs from HT29-18N2 cells, at various time intervals, after 8 h stimulation with two reactogenic cholera vaccine strains grown inside Transwell devices to avoid epithelial cells-vibrios interactions. IM-8h: HT29-18N2 cultures stimulated for 8 h with the induction mixture (positive inductor) added inside Transwell devices. Sizes are indicated in base pairs (bp).*

DISCUSSION

Epithelial cells are the initial sites of interactions of the host with invasive and noninvasive microbial pathogens. Following bacterial injury, cells from different epithelia can produce proinflammatory mediators that include IL-1α and β, IL-6, IL-8, TNF-α, GM-CSF and many others, suggesting that those cells serve as sensors for the bacterial infection to the host and provide signals for the initialization of the inflammatory and immune reactions.[26,18,27] Here it was examined the induction of a wide range of inflammatory mediators in HT29-18N2 cells exposed to reactogenic and nonreactogenic vaccine strains. The results demonstrated that strains (JBK70 and 81) and (CVD103HgR and 638) that were reactogenic and nonreactogenic in volunteers studies, respectively,[10,11,28] induced a differential array of three proinflammatory mediators mRNA (IL-8, GM-CSF and TNF-α) in HT29-18N2 cells, which was always higher for reactogenic vaccine strains. The absence of TNF-α transcripts and the lower mRNA expression in epithelial cells exposed to heat-killed and nonreactogenic live vaccines, respectively, indicate that TNF-α, which is an important inflammatory activator, could play and important role in the inflammatory component of reactogenicity. Of note, *V. cholerae*-infected intestinal cells did not secrete GM-CSF although its mRNA is expressed, suggesting a delayed secretion due to some kind of regulation for this cytokine in the HT29-18N2 cell line. In fact, intestinal epithelial cells, including the HT29 cell line, express GM-CSF receptors at levels similar than those seen on monocytes,[29] which in turn have been reported to down-regulate GM-CSF secretion by a up-regulating expression of its receptors.[30] The differential proinflammatory expression pattern reported here for reactogenic and nonreactogenic strains, add new evidences in favor of a stronger inflammatory component found in the diarrhea of volunteers that orally received a reactogenic live cholera vaccine.[15] Additionally, the lower induction of proinflammatory mediators observed for heat-killed vaccine stains are also in concordance with the results obtained in humans for dead cholera vaccines, in which untoward reactions did not occur.[31,32] Moreover, the epithelial proinflammatory response was not induced in Trasnswell experiments. Taken together, these results demonstrate that cholera vaccine/intestinal epithelial cell interactions are needed to elicit an inflammatory signal expression in the HT29-18N2 cell line, which is differential and always higher in epithelial cells exposed to reactogenic vaccine strains and suggest that vibrio-associated factor(s) alone or in combination with a bacterial soluble factor(s), produced during vibrio/HT29-18N2 interactions, are responsible for the highest proinflammatory induction observed with reactogenic vaccines. Interesting, nonmotile mutants of *V. cholerae*, which are supposed to have a reduced capacity to reach epithelial cells, had lower reactogenicity compared with parental motile vaccine strains in volunteer studies.[14] The HT29-18N2 response observed here to cholera vaccine strains, have the potential to orchestrate the onset of a mucosal inflammatory response, which in turn may induce profuse diarrheas as seen in reactogenic live cholera vaccine recipients.[10-13] Further experiments, using purified putative reactogenic factors and their *V. cholerae* isogenic mutants are needed to determine the triggering factors for this differential epithelial cytokine response.

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