Development of Microbial-Human Enterocyte Interaction: Cholera Toxin

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ABSTRACT

Diarrhea in infants and children is a major health hazard worldwide. Certain toxigenic diarrheas occur more commonly and are manifested more severely during the neonatal period. We have previously studied the regulation of cholera toxin-induced secretion in animal models during development. In those studies we have shown that cholera toxin stimulates a much greater secretion by immature compared with mature small intestine, and the mechanism appears to be an up-regulation of postreceptor signal transduction molecules (adenyl cyclase and $G_{s\alpha}$) leading to an elevated cAMP level. In this study, using experimental models of human intestinal development (fetal cell lines, a micro-Ussing chamber, organ cultures, and fetal intestinal xenograft transplants), we provide preliminary evidence that cholera toxin induces an enhanced secretion mediated in part by a developmental up-regulation of the cAMP response in immature *versus* mature human small intestine. Additional studies are needed, however, to further define whether other developmental

events (e.g. receptor expression) also regulate cholera toxin-enterocyte-enhanced interaction. Nonetheless, this approach to determining the role of development in the pathophysiology of cholera in infants may help in strategies to prevent and treat this condition and other age-related intestinal infectious diseases. (*Pediatr Res* 54: 212–218, 2003)

Abbreviations

CT, cholera toxin
 I_{sc} , short circuit current
T84, a human colonic cancer cell line
H4, a primary human fetal crypt small intestinal cell line
tsHFIE, a temperature-sensitive, conditionally immortalized human fetal intestinal epithelial villous cell line
PGE₂, prostaglandin E₂
SCID, severe combined immunodeficiency
CFTR, cystic fibrosis transmembrane conductance regulator

Diarrheal disease constitutes one of the major causes of morbidity and mortality in infants and young children on a global scale (1). We know from clinical and laboratory studies that certain toxigenic diarrheas occur more commonly and are manifested more severely in the neonatal period (2). Toxigenic diarrhea is caused by a bacterial exotoxin binding to an enterocyte receptor and the activation of a signal transduction pathway leading to enhanced secretion and decreased fluid absorption (3). Our laboratory has had a long-standing interest in developmental factors affecting CT-induced diarrhea as a paradigm for microbial-epithelial “cross talk” in the developing small intestine. We have provided evidence that there is a

striking difference in host responsiveness to CT as measured by fluid accumulation in intestine loops from preweaned rats when compared with loops from mature (postweaned) animals (4). A similar increased sensitivity to *Escherichia coli* heat-stable enterotoxin in immature animals and man was reported by Cohen *et al.* (5). The mechanism of this excessive secretory response to CT in the immature rat intestine was not because of an increased number of toxin receptors on the apical surface as was expected and could only partially be explained by an increased receptor affinity (6). We subsequently demonstrated increased enterocyte sensitivity to CT-induced adenylate cyclase activation as the basis for cAMP-mediated enhanced secretion (7). We further reported that pretreatment of the immature rat intestine with cortisone, an intestinal trophic factor, caused a change in CT-induced secretion toward a mature response (4). These results suggested that an immaturity of enterocytes and their signaling machinery may be the basis for the enhanced developmental CT response.

Despite the advances in understanding the molecular mechanisms of action of CT, cholera remains a major international

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health problem. Furthermore, the CT-induced secretory effect on the gut has proven to be more complex than originally envisioned (8). In addition, extrapolating events (mechanisms) of response from animal models of development to presumed events in the developing human intestine may not be valid. Accordingly we have begun to study CT interaction with the developing human small intestine to determine whether a similar effect of CT existed in the human intestinal immature gut and whether the effect was mediated in part by developmental regulation of postreceptor events. This approach should be helpful in defining the developmental regulation of human enterocyte interaction with luminal pathogens and their exotoxins in general, as well as in establishing its relationship with the pathogenesis of age-related gastrointestinal infectious diseases.

Thus, in this study, we have extended the observations reported on the interaction of CT with animal models of developing small intestine (2, 4, 7) to begin to define CT interaction with the developing human gut. This laboratory has either established or validated several experimental models of the developing human small intestine to study their interaction with luminal microorganisms and their exotoxins. These models include a micro-Ussing chamber technique to study Cl^- secretion (9), two human fetal small intestinal cell lines (10, 11) to define postreceptor signaling events, and organ culture (12) and a human fetal intestine xenograft model (13) to confirm the initial results made in other models. Using these techniques collectively we now provide the first preliminary evidence that the immature human small intestine also responds excessively to CT and the excessive response maybe caused in part by a developmental up-regulation of postreceptor signal-transduction events, (e.g. cAMP).

METHODS

Reagents. CT, PGE_2 , and 5-nitro-2-(3-phenylpropylamino) benzoic acid were obtained from Calbiochem Laboratory (San Diego, CA, U.S.A.). Tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Other chemicals, if not specified, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals. Female Sprague-Dawley rats were purchased from Taconic (Germantown, NY, U.S.A.) between 16 to 18 days of gestation. Animals were kept in an institutional animal facility with a 12-h light-dark cycle. They were fed rat chow and water *ad libitum*. This study has been approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Experiments were performed when the litters were 2 wk of age for the preweaned group and 4 wk of age for the postweaned group. Half of the litters were sacrificed at 2 wk and the other half weaned at 3 wk and sacrificed at 4 wk. Adult animals were sacrificed at 10 wk.

I_{sc} measurements. The I_{sc} across the intestinal epithelium of rat small intestine was measured according to procedures previously described (14) with modifications. Briefly, preweaned rat small intestinal mucosa was directly mounted on micro-Ussing chambers (Physiologic Instruments, San Diego, CA, U.S.A.) with an exposed area of 0.22 cm^2 , and postweaned

and adult rat small intestines were gently stripped of the serosal layer without disrupting the mucosal surface and then mounted on chambers with an exposed area of 0.4 cm^2 . Human fetal small intestine obtained from 20-wk-old fetuses were stripped of the serosal layer and mounted onto a micro-Ussing chamber as described above. Biopsy tissues obtained from pediatric patients during endoscopy were carefully mounted onto a micro-Ussing chamber under the dissecting microscope and bathed in an oxygenated buffer as described (9). The results of micro-Ussing chamber studies were included only if the fetal intestinal or intestinal biopsy specimens from infants and children were morphologically normal and the fetus, infant, or child was free of gastrointestinal disease diagnosis. Both apical and basolateral surfaces were bathed in Ringer's solution. The composition of the media used was (in mM): NaCl, 113; KCl, 4.7; MgSO_4 , 1.2; CaCl_2 , 1.9; NaHCO_3 , 25; and glucose, 12.1. The bathing solutions were maintained at 37°C and oxygenated with 95% O_2 and 5% CO_2 to optimize oxygen supply to the tissue and to maintain the pH at 7.4.

After mounting, each preparation was connected to a voltage clamp (Physiologic Instruments) with current and electrodes placed on either side. To compensate for fluid resistance, preparations were clamped to zero potential difference and were allowed to equilibrate for 20–40 min until a steady-state basal I_{sc} was achieved. The apical surface was incubated with CT ($1 \mu\text{g/mL}$) or buffer alone, and the I_{sc} was measured at 5-min intervals for 60 min. In each case, the control (medium alone) response was compared with the response achieved in the presence of CT. In some experiments to assess ongoing tissue viability, glucose (25 mM) was added to the apical surface of the tissue at the end of CT treatment, and the I_{sc} change was recorded.

Maintenance of cell cultures. H_4 cells, developed in our laboratory (10), are nontransformed human fetal primary small intestinal epithelial cells that are comparable to the IEC-6 rodent crypt cell line (15). H_4 cells were routinely maintained in plastic culture flasks with Dulbecco's modified Eagle medium, supplemented with 10% FCS, 20 mM L-glutamate, and human recombinant insulin (0.5 U/mL). A temperature-sensitive, conditionally immortalized human fetal small intestinal cell line [tsHFIE (11)] was generously supplied by Dr. Andrea Quaroni, Department of Biology, Cornell University (Ithaca, NY, U.S.A.). This human fetal enterocyte cell line was originally developed and characterized by Quaroni and Beaulieu (11) using a stable transfection of 13-wk human fetal enterocytes with a temperature-sensitive large T antigen DNA from the simian virus attached to an enterocyte-specific promoter and a retroviral vector. At 33°C these cells function as transformed enterocytes, and at 39°C the T antigen is inactivated and cells undergo differentiation and function as primary villous enterocytes. The tsHFIE cells were routinely maintained in Dulbecco's modified Eagle medium, supplemented with 10% FCS and 20 mM L-glutamate at 33°C on 12-well tissue culture plates. On confluency, cells were changed to the media containing $1 \mu\text{M}$ dexamethasone and incubated at 39°C for 1 wk to inactivate the large T antigen and induce differentiation. T_{84} cells, obtained from American Type Culture Collection (Washington, D.C, U.S.A.), were routinely maintained

in plastic culture flasks containing minimal essential medium-F12 HAM, supplemented with 10% FCS and 20 mM L-glutamate. T₈₄ cells were trypsinized after becoming confluent using a 0.25% trypsin-0.5 mM EDTA solution and reseeded at one fifth the original density onto collagen-coated filter inserts (Transwell, Corning, NY, U.S.A.). T₈₄ cells form a polarized monolayer when grown on these filters.

cAMP extraction from human fetal small intestine xenografts and biopsy samples. Human fetal small intestine xenografts were supplied by the Xenograft Core Facility in our laboratory. Briefly, fetal small intestine from 16- to 20-wk fetuses was transplanted s.c. into SCID mice and kept in a pathogen-free environment for 6 mo as previously reported (13). We have shown in several recent publications (16, 17) that fetal tissue transplanted into SCID mice reepithelializes and matures in a manner similar to that in the intrauterine environment as determined by microvillous disaccharidases and other enzymes. Accordingly, when studied the xenograft small intestinal samples are as viable as intestinal biopsy samples. Tissues were obtained by carefully removing all the serosal and muscle tissues surrounding the intestinal mucosa and cut into small pieces using a surgical blade. Human biopsy tissue from children undergoing diagnostic endoscopy for abdominal complaints was obtained. If the tissue appeared normal under the dissecting microscope and no gastrointestinal diagnosis was made, it was processed for study and maintained in 95% oxygenated culture media until transferred to the laboratory. Only data from specimens subsequently noted to be normal morphologically were included in the study. Fetal tissue and intestinal biopsies were obtained with the approval of the Partners Human Study Committee and with parental signed permission. After exposure to CT or media under conditions previously used in cells, tissues were homogenized with a handheld polytron. The homogenized mixture was spun briefly, the supernatant was loaded onto trimethylaminopropyl SAX minicolumns (Amersham, Little Chalfond, Buckinghamshire U.K.), and cAMP was extracted with methanol according to the manufacturer's recommendation.

Measurement of intracellular cAMP levels. Cell lines were grown to confluence and incubated with various concentrations of CT in Hanks' balanced salt solution for 1 h. Tissues either from xenografts or biopsies were cut into small pieces and incubated with CT or media alone for 2 h. At the end of incubation period, cells were washed three times with cold PBS, and 500 μ L of lysis buffer (Amersham) was added to each well. The cell lysates were collected and stored at -20°C if not measured immediately. Tissues were snap-frozen in liquid nitrogen and stored at -20°C until further analysis. Cellular cAMP levels were measured using a commercially available enzyme immunoassay kit (Amersham Biotrak), normalized with the total protein content and expressed as picomoles per milligram protein. Protein concentrations were determined using a Bio-Rad protein assay kit with BSA as the standard (BioRad, Hercules, CA, U.S.A.).

Statistics. Data are presented as the mean $\pm$ SEM. Statistics were performed with StatView (version 4.5; SAS Institute, Cary, NC, U.S.A.) using either an unpaired *t* test or ANOVA. A *p* < 0.05 was considered statistically significant for the *t* test

and *p* < 0.0033 for the Dunn test (a cutoff of *p* < 0.0033 is mandated by the StatView program for this test).

RESULTS

CT-Induced Chloride Secretion in the Developing Small Intestine

Rat studies. In this study, we have begun to use a micro-Ussing chamber technique (9) to measure chloride secretion in human small intestinal models. To validate this technique as an appropriate measurement of the CT response, we first tested it in preweaned and postweaned rats as a model for age-dependent differences in chloride secretion in response to CT (Fig. 1). The addition of CT compared with control media to the apical membrane surface of preweaned rats resulted in a significant increase in I_{sc} (Fig. 1A) with time, reaching a peak at 60 min. As reported previously in the *in vivo* loop model (4), CT induced a significantly higher secretory response in preweaned compared with postweaned and adult rats as measured by I_{sc} during 60 min (Fig. 1B). The I_{sc} change was noted to diminish after the addition of the chloride-channel inhibitor, 5-nitro-2-(3-phenylpropylamino) benzoic acid, to the bathing solution (data not shown). These studies confirmed that preweaned rats were more responsive to CT than postweaned rats using the micro-Ussing chamber technique. To further define an age-related effect on chloride secretion in preweaned and postweaned rats, PGE₂ (10 μ M), known to induce chloride secretion from intestinal epithelia, was added to the solution bathing the basolateral surface of micro-Ussing chambers (Fig. 1B, insert). A PGE₂-stimulated I_{sc} response had a mean increase of $15.9 \pm 4 \mu\text{A}/\text{cm}^2$ in preweaned compared with $15.4 \pm 9 \mu\text{A}/\text{cm}^2$ in postweaned rats, suggesting no age-dependent difference.

Human studies. Having validated the micro-Ussing chamber technique to measure CT-induced chloride secretion in preweaned and postweaned rats, we next performed comparable studies on fetal (immature) small intestine and on (mature) small intestinal biopsies from children. In six separate experiments, fetal intestine from 16- to 20-wk aborted fetuses was compared with small intestinal biopsies from infants or children exposed to CT (1 $\mu\text{g}/\text{mg}$) apically. Each comparative experiment showed a greater rise in I_{sc} with time for fetal tissue compared with tissue from infants or children. Inasmuch as each experiment was a unique observation, the six experiments cannot be compared statistically. Accordingly, Figure 2A is a representative example of these six observations on I_{sc} . To assure continued viability of the biopsy after CT stimulation, we exposed tissue to glucose. Figure 2B shows the I_{sc} after glucose infusion. These experiments strongly suggest that the immature human small intestine, like that of preweaned rats, is more sensitive to CT than the mature intestine.

cAMP Response to CT

Human cell lines. Having demonstrated a excessive developmental secretory response to CT in human immature *versus* mature small intestine similar to that noted in our animal models using micro-Ussing chambers, we next determined

Figure 1. A, effect of CT on percent change of I_{sc} in preweaned rat small intestine mucosa. CT (●; 1 $\mu\text{g}/\text{mL}$) was added to apical membranes mounted on micro-Ussing chambers. Control (○) tissues indicate change in I_{sc} with time. Secretion is expressed as a change in I_{sc} from baseline. Each point represents the mean $\pm$ SEM ($n = 15$). * $p < 0.05$, ** $p < 0.01$. B, effect of CT on I_{sc} percent change of small intestinal mucosa of preweaned (■; $n = 15$), postweaned (▨; $n = 10$), and adult (□; $n = 10$) rats. Rat small intestinal mucosa was mounted onto micro-Ussing chambers and apical surface was exposed to CT (1 $\mu\text{g}/\text{mL}$). Chloride secretion is expressed as I_{sc} change from baseline. Each bar is the mean $\pm$ SEM. * $p < 0.0033$ by ANOVA Dunn test. Inset, response after PGE₂ (10 μM) stimulation. There is no difference in the I_{sc} response between preweaned (■) and postweaned (□) rat small intestine after PGE₂ stimulation.

whether the excessive secretory response was in part related to a post-CT receptor signal-transduction reaction as previously reported in animal models (6, 18). Because CT-induced chloride secretion is mediated via the cAMP pathway, we determined levels of cAMP in the two human fetal cell lines (H₄ and tsHFIE) compared with a more mature human transformed cell line, T₈₄. In Figure 3, we show a significant difference in the cAMP response to CT between H₄ and tsHFIE (immature human enterocytes) and T₈₄ cells (representative mature human enterocytes). CT was noted to stimulate a dose-dependent increase of cellular cAMP production after 1-h exposure in these cells compared with media alone. Moreover, there was a significantly higher level of CT-induced cAMP in H₄ and tsHFIE cells compared with T₈₄ cells (Fig. 3A). The basal levels of cellular cAMP in H₄, tsHFIE, and T₈₄ were $17.8 \pm$

Figure 2. A, effect of CT on I_{sc} on percent change of fetal duodenal intestinal tissue and in normal duodenal biopsies from children. A 20 week-old fetal duodenal mucosa (—●—) and duodenal biopsies from a 4-year-old (—▲—) and an 8-year-old (—■—) child were mounted onto a micro-Ussing chamber and exposed to CT (1 $\mu\text{g}/\text{mL}$). Each point is expressed as a change in I_{sc} as a percent of baseline. Six experiments that compared 20-wk-old fetal small intestine with various aged pediatric small intestine biopsies were performed independently. This tracing is representative of these six experiments. B, test of sustained tissue viability in micro-Ussing chamber after CT stimulation demonstrating the response of small intestinal biopsies from 8-year-old child mounted in a micro-Ussing chamber to glucose (25 mM) after the test period with (—▲—) or without (—○—) CT stimulation (60 min). The I_{sc} response to glucose suggests that the intestine studied remains viable during the test period.

2.8, 11.8 ± 3.3 , and 6.0 ± 1.1 pmol/mg, respectively. The mean difference in cAMP levels after CT stimulation between H₄ and T₈₄ cells or tsHFIE and T₈₄ cells was 88.7 and 86.0 pmol/mg ($p < 0.01$) with 0.1 $\mu\text{g}/\text{mL}$ CT, 122 and 142.7 pmol/mg ($p < 0.01$) with 1 $\mu\text{g}/\text{mL}$ CT, and 59.8 and 129 pmol/mg ($p < 0.01$) with 10 $\mu\text{g}/\text{mL}$ CT. When the fold increase in cAMP response was measured at each concentration of CT, the same differences between H₄ or tsHFIE versus T₈₄ was maintained (Fig. 3B). In contrast, we used forskolin to directly activate adenylate cyclase in the cell, and cAMP production after forskolin stimulation was the same in both H₄ and T₈₄ cells (366.03 ± 186 versus 359.34 ± 177 pmol/mg),

Figure 3. A, effect of CT on intracellular cAMP production in immature vs mature human enterocytes. Human fetal small intestinal cell lines [H₄ (▨) and tsHFIE (□) cells] as well as T₈₄ cells (■) were stimulated either with various concentrations of CT or media alone for 1 h. Cellular cAMP levels were measured and expressed as picomoles per milligram of total protein. * $p < 0.05$, ** $p < 0.01$. B, effect of CT on intracellular cAMP as determined by a fold increase in H₄ (▨), tsHFIE (□), and T₈₄ (■) cells. All cell lines were stimulated either with various concentrations of CT or with media alone for 1 h. Cellular cAMP level was measured and expressed as a fold increase over baseline. There is a consistently higher fold increase in cellular cAMP in H₄ and tsHFIE cells compared with T₈₄ cells.

suggesting a specific CT-induced developmental response. We feel justified in comparing the CT response in H₄ and tsHFIE cells with that of confluent T₈₄ cells because a similar response was noted in fetal *versus* infant or child small intestine as a comparison. In previous studies we have shown that H₄ and tsHFIE cells respond differently to an inflammatory stimulus compared with Caco-2, T₈₄, or HT29 cells (19, 20), and the same difference was noted when comparing intestinal biopsy samples from fetal and infant or child small intestine.

Organ cultures and xenografts. To confirm the differences found in cAMP activation after CT stimulation between immature and mature cell lines, tissues from human fetal small intestine xenografts exposed to CT were examined for cAMP levels and compared with those obtained from small intestinal biopsies in infants or older children exposed to CT. Our data showed CT induced a cAMP accumulation in these tissues, and that the CT-induced response was greater in fetal small intestine

xenografts compared with small intestine biopsies from children. Figure 4 shows the results of these studies.

DISCUSSION

These studies for the first time provide direct preliminary experimental evidence that CT stimulation results in an excessive chloride secretion in immature compared with mature human small intestine. In addition, the studies suggest that secretion appears to be developmentally regulated and in part related to an excessive cAMP production in response to CT. These observations made in the human fetal small intestine correspond to comparable observations previously reported from this laboratory (4, 6, 7, 18) in animal models. A similar enhanced intracellular response to bacterial endotoxin in the immature (fetal) human small intestine has previously been reported in studies involving an exogenous bacterial inflammatory mediator, lipopolysaccharide and an endogenous mediator, IL-1 α . In these studies a much greater IL-8 response was noted in H₄ cells compared with Caco-2 cells, suggesting a developmentally regulated intracellular activation of the cytokine (20). These observations were validated by a similar IL-8 differential response to lipopolysaccharide and IL-1 α in organ cultures of fetal and infant or child intestine. This previous observation adds credence to the observations made in this study.

To provide these observations, we have had to use various models for the developing small human intestine. Two human

Figure 4. Effect of CT on intracellular cAMP production in fetal xenograft organ cultures vs a biopsy organ cultures from children at various ages. Human intestinal samples either from human fetal small intestine xenografts ($n = 4$) or pediatric small intestine biopsies ($n = 4$) were incubated with CT (▨) or media alone (□) for 2 h. Cellular cAMP was extracted using a solid-phase method and measured. The level of cAMP is expressed as femtomoles per milligram total protein. Data show an increase in cAMP production after CT stimulation in both tissues, but the CT response is much greater in fetal small intestine xenograft organ cultures. This observation confirms the studies in intestinal cell lines.

fetal small intestinal cell lines (H_4 and tsHFIE) have been used in this laboratory to determine *in vitro* responses of the developing intestine to microbial flora and their toxins. The cellular cAMP responses to CT in both cell lines (representative immature enterocytes) have been compared with confluent T_{84} monolayers (representative mature enterocytes) in this study, and the comparison was validated using organ cultures of immature and mature small intestine. To test for chloride secretion in response to CT stimulation in immature *versus* mature human small intestine, a previously reported micro-Ussing chamber technique (9) was validated. This technique enabled us to use tiny quantities of an intestinal mucosa biopsy to measure enterocyte resistance and I_{sc} when CT was applied to the mucosal surface. A change in I_{sc} corresponds to a quantitative measurement of chloride secretion.

To validate this technique as an accurate developmental measure of the CT response, we used small intestinal mucosa from preweaned and postweaned rats in the micro-Ussing chamber. Using *in vivo* small intestinal loops, we have previously reported (4) that fluid secretion in preweaned small intestine was much greater than in postweaned small intestine. In these studies a similar excessive response to CT stimulation (increased I_{sc} ; Fig. 1) was noted in preweaned *versus* postweaned rat small intestine. Based on this validation, we believed that this technique could be used to study the developmental secretory response in human small intestine.

Furthermore we have validated an established a technique for xenograft transplantation of fetal human intestine into the s.c. capsule of SCID mice (13). Once small intestinal loops of fetuses of gestational age 16–20 wk are transplanted into the s.c. capsule, a reepithelialization occurs and they develop in a manner similar to that predicted for the third trimester of human gestation *in utero* (16, 17). Fetal human xenografts can be isolated and exposed to CT and then used to measure cAMP accumulation. Therefore in this study, we have used models of human small intestinal development, (*e.g.* intestinal organ culture and fetal small intestinal xenograft transplantation) to confirm the noted increase in cAMP response to CT stimulation observed in fetal human *versus* mature human cell lines (Figs. 3 and 4). We believe the collective use of these techniques provides compelling evidence that the fetal enterocyte secretory response to CT stimulation is accentuated *versus* the mature enterocyte and at least in part is developmentally regulated vis-à-vis an enhanced cAMP response. As we have not measured CT receptor number and affinity or developmental expression of CFTR in these studies, we cannot conclude that this observation is the only explanation for the enhanced CT-stimulated secretion in immature human intestine.

In the last decade a number of important studies have suggested that CT can mediate chloride secretion by means other than the classically described pathway (2). For example, CT can interact with receptors on enteroendocrine cells to cause the release of 5-hydroxytryptamine and prostaglandins (*e.g.* PGE_2). By interacting with the enteric nervous system, these mediators can affect release of the neuropeptide vasoactive intestinal polypeptide at enteric nerve endings juxtaposed to crypt cell enterocytes. Vasoactive intestinal polypeptide may in turn activate cAMP through its own basolateral receptor to

mediate chloride secretion (21–23). Alternatively, 5-hydroxytryptamine and PGE_2 may directly stimulate chloride secretion through an as yet unidentified pathway in enterocytes (24). These alternative mechanisms of CT action may explain the extent of the muted secretory responses we observed using the micro-Ussing chamber compared with that reported using an *in vivo* loop model in rat small intestine. However, studies have demonstrated a nonneural effect of CT on chloride secretion in isolated small intestinal mucosa *in vitro* (25). Thus the micro-Ussing chamber technique is a valuable technique that permits us to characterize a single CT-mediated chloride secretion process. Furthermore, the micro-Ussing chamber will allow us to quantify chloride secretion in response to CT from human small intestine biopsy tissue and human fetal intestine xenografts as models of human gut development. By using these techniques collectively, including *in vitro* cellular studies, we should be able to characterize the developmental mechanism(s) contributing to the differentiated CT response in immature compared with mature human enterocytes. Preliminary data using PGE_2 (Fig. 1B) in this study to compare effects in preweaned and postweaned rats suggest that the PGE_2 -stimulated chloride secretion is not developmentally regulated. Additional, more-comprehensive studies are needed to finalize this observation.

Previous studies have shown that developmental changes in membrane structure that influence binding affinities but not receptor densities may, in part, contribute to the increased sensitivity of suckling rats to CT (6). However, the relatively small differences in binding to cell surface receptors suggested that a subsequent postreceptor mechanism may account for a greater part of the observed developmental difference noted in the cellular response (7). CT induces secretion by activation of the adenylyl cyclase signaling cascade, resulting in increased levels of the cellular second messenger cAMP (26). The increase in cAMP and activation of the chloride channel is thought to be the principal mechanism for the pathologic actions of CT. Heretofore, most of the studies on CT activation of adenylate cyclase have been carried out in cells other than intestinal enterocytes or in animal enterocytes. A study such as this, which addresses the cellular mechanism of a developmentally different CT response in human enterocytes, seems particularly advisable as little is known of the mechanisms by which human immature enterocytes respond to CT. As stated, we used two well-established human fetal intestinal epithelial cell lines, H_4 and tsHFIE, and compared responses to the T_{84} cell line as prototypes of immature and mature human enterocytes. We have found that, when incubated with CT, H_4 cells generate a significantly higher level of intracellular cAMP compared with T_{84} cells (Fig. 3). Furthermore the differences in intracellular cAMP levels are not the result of a generalized reduced capacity of adenylate cyclase to convert ATP to cAMP because both cell types have shown a similar level of cAMP increase after incubation with a supramaximal dose of forskolin, a generic cAMP ligand. This preliminary observation suggests that the developmental response of cAMP to CT stimulation may be specific and not a generalized overreaction to all cAMP-stimulating ligands. Further, more-extensive studies are needed using other ligands to confirm this observation.

Activation of the apical chloride ion channel is stimulated by agonists that increase the cellular cAMP level. The increase in cAMP results in activation of a cAMP-dependent protein kinase A, which modulates actions of target proteins by phosphorylating cellular substrates, including the CFTR (27). CFTR is the most clearly identified chloride channel in the intestinal epithelium. The importance of this protein is evident from the observation that the secretory responses to many agonists including CT is absent in cystic fibrosis (28, 29). Because both H₄ and T₈₄ cells express CFTR by reverse transcription-PCR and by immunoprecipitation (data not shown) and other investigators have shown there is a direct correlation between an increased level of cellular cAMP and CT-induced chloride secretion in an intestinal epithelia HT29 cl 19A cell monolayer (30), we have reason to believe the elevation of cAMP is correlated with chloride secretion in response to CT in these two cell lines. We speculate that the secretory response to CT in H₄ cells is more sensitive than that of T₈₄ cells.

It is possible that the excessive secretory response in fetal enterocytes reported in this study may also be a result of other increased developmentally regulated intracellular steps, including the protein kinase A response or an increased expression of CFTR developmentally. We plan experiments to determine whether additional developmentally regulated intracellular processes contribute to the enhanced CT-stimulated secretion.

CONCLUSIONS

This study represents an initial preliminary observation in human fetal cells that the CT-stimulated secretory response is developmentally regulated and involves in part the cAMP signal transduction pathway. What remain to be determined are the precise developmental events that regulate the maturation of cellular signaling and the response to CT. From the preliminary data presented, the enhanced cAMP activation appears to be CT specific. In a more global sense, the use of CT as a probe to examine developmental regulation of secretion in human fetal enterocytes may represent an important paradigm for our understanding the development of microbial-human enterocyte interactions in general, as previously reported from this laboratory (20), and provide insights into mucosal barrier function in the extrauterine environment. Moreover, these studies may lead to a better understanding of the pathophysiology of cholera toxigenic diarrhea in infants and may better enable us to prevent or treat this and other age-related intestinal infectious diseases.

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