Testing the Efficacy and Toxicity of Adenylyl Cyclase Inhibitors against Enteric Pathogens Using *In Vitro* and *In Vivo* Models of Infection[▽]

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Enterotoxigenic *Escherichia coli* (ETEC) produces the ADP-ribosyltransferase toxin known as heat-labile enterotoxin (LT). In addition to the toxic effect of LT resulting in increases of cyclic AMP (cAMP) and disturbance of cellular metabolic processes, this toxin promotes bacterial adherence to intestinal epithelial cells (A. M. Johnson, R. S. Kaushik, D. H. Francis, J. M. Fleckenstein, and P. R. Hardwidge, J. Bacteriol. 191:178–186, 2009). Therefore, we hypothesized that the identification of a compound that inhibits the activity of the toxin would have a suppressive effect on the ETEC colonization capabilities. Using *in vivo* and *in vitro* approaches, we present evidence demonstrating that a fluorenone-based compound, DC5, which inhibits the accumulation of cAMP in intoxicated cultured cells, significantly decreases the colonization abilities of adenylyl cyclase toxin-producing bacteria, such as ETEC. These findings established that DC5 is a potent inhibitor both of toxin-induced cAMP accumulation and of ETEC adherence to epithelial cells. Thus, DC5 may be a promising compound for treatment of diarrhea caused by ETEC and other adenylyl cyclase toxin-producing bacteria.

Diarrheal diseases caused by enteric pathogens such as enterotoxigenic *Escherichia coli* (ETEC) or *Vibrio cholerae* remain a major cause of morbidity and mortality worldwide (25, 31, 34). ETEC, a pathogen of increasing frequency in the United States, is a leading cause of traveler's diarrhea (36). Prevention of diarrhea caused by these toxigenic organisms, by virtue of improved hygiene and provision of sanitation and water treatment, often is impractical in most developing countries, where the morbidity and mortality rates are highest (37). ETEC and *V. cholerae* produce the heat-labile toxin (LT) and cholera toxin (CT), respectively, and both toxins display ADP ribosylation activity, which results in increased chloride and water efflux into the intestinal lumen, leading to significant volumes of watery diarrhea (25). Interestingly, recent studies have confirmed prior observations indicating that enterotoxins, such as LT and CT, enhance enteric bacterial colonization and pathogenicity (reviewed in reference 8). Anti-toxigenic compounds have been shown to decrease morbidity and mortality of diseases caused by other toxin-producing bacteria (18, 29). Therapy using anti-toxigenic compounds is therefore an area of great interest. Identification of a new class of drugs that afford selective anti-toxigenic activities would constitute a highly desired compound useful for future therapy; however, these drugs need to be experimentally validated by first testing

efficacy, bioavailability, and the absence of toxicity in relevant animal models.

We have previously shown that prostaglandin E₂-histidine (PGE₂-L-histidine) and prostaglandin E₂-imidazole (PGE₂-imidazole) adducts significantly reduced CT-induced fluid loss and cyclic AMP (cAMP) accumulation in the murine ligated small intestinal loop model (21). These and other derived adducts have been shown to act on ETEC LT and on the edema factor (EF) produced by *Bacillus anthracis* (15). Our recent progress has resulted in the development of structurally stable compounds that inhibit toxin-induced accumulation of cAMP in *in vitro* cell culture assays (3). Our studies have shown that although some of these compounds are extremely active *in vitro* and showed reduced fluid accumulation in the murine model of experimental cholera, they were also toxic, causing destruction and bleeding of the intestinal lumen (unpublished data). Further, we have also identified nontoxic compounds that inhibited fluid accumulation caused by CT injection in a murine intestinal loop model (unpublished data). The mouse intestinal loop assay, however, is somewhat artificial because the intestine remains ligated, preventing normal flow of the intestinal contents, which is important in testing the efficacy of the compound. Because one of our goals is to identify a compound(s) that can protect against diarrhea caused by a whole organism in a natural setting and not just by purified toxins, in the current study, we utilized a murine model that mimics more closely the infection route used by ETEC (2, 6, 21, 24, 28). We hypothesized that the murine model of experimental diarrhea using ETEC bacteria not only was an appropriate way to distinguish between those compounds that are effective against the toxin but might also help to identify possible toxic

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effects *in vivo*. This method provided the advantage of being minimally invasive; the flow in the intestine was not interrupted, and the method provided the advantage of being able to determine that the organism was capable of colonizing the small-intestine mucosa while being affected by the tested compound. Using this method, we established evidence demonstrating that a novel toxin inhibitor, 3-[(9-oxo-9H-fluorene-1-carbonyl)-amino]-benzoic acid, called DC5 (3, 4), which inhibits the accumulation of cAMP in cultured cells, significantly decreased the fluid loss caused by the LT and reduced the colonization activity of ETEC strains.

MATERIALS AND METHODS

Bacterial strains, reagents, and growth conditions. Bacterial strains used in this study include ETEC strains H10407 and DEC7A, enteropathogenic *E. coli* (EPEC) O127:H6 (isolate E2348/69), enterohemorrhagic *E. coli* (EHEC) O157:H7 (isolate 86-24), enteroaggregative *E. coli* (EAEC) O42, *Salmonella enterica* serovar Typhimurium 157, *Shigella flexneri*, and *V. cholerae* 569B. Strains were routinely grown overnight in Luria-Bertani (LB) medium at 37°C, and prior to the animal infection, the bacterial culture concentration was estimated spectrophotometrically and samples were diluted to the required working concentration. DC5 for these studies was synthesized in-house from commercially available precursors. The compound was dissolved in a minimal volume of anhydrous dimethyl sulfoxide (DMSO, usually to 100 mM) and then diluted to 10 mM into phosphate-buffered saline (PBS) buffer plus two molar equivalents of NaOH. Samples were then diluted 10× (1 mM) or 100× (0.1 mM) into cell culture or other medium before use in cell culture assays or for animal studies. Appropriate controls indicated that the end concentration of DMSO (1% DMSO) had no effect on the assays. The stock of LT toxin (kindly provided by J. Peterson) was suspended in 1 ml of sterile PBS (1 mg/ml), and the LT toxin in solution was transferred to a freezer vial and stored at -80°C until use.

***In vitro* cell-based cAMP assay.** (i) **Cell propagation.** Murine monocyte/macrophage cells (RAW 264.7) were propagated in T75 flasks containing Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Herndon, VA) at 37°C with 5% CO₂. The culture media contained 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, and 100 µg/ml penicillin/streptomycin.

(ii) **cAMP assays.** Cells were plated at a cell density of 5×10^5 cells/ml in 48-well assay plates and incubated overnight. The next day, RAW 264.7 cells were incubated with various concentrations of the inhibitors (from 100 µg/ml to 0.1 µg/ml) in the presence of purified protective antigen (PA, 2.5 µg/ml), edema factor (EF, 0.625 µg/ml), and 50 µM 3-isobutyl-1-methylxanthine (IBMX) (BIOMOL, Plymouth Meeting, PA) in triplicate for 4 h. To determine the amount of cAMP released, we used the Correlate-EIA direct cyclic AMP kit (Assay Designs, Ann Arbor, MI) to quantitatively determine amounts of extracellular cAMP from supernatants in duplicate.

Determination of IC₅₀. Three experiments in triplicate were performed, the average values were used to construct dose-response curves, and the 50% inhibitory concentrations (IC₅₀s) were calculated (Tablecurve 2D; AISN Software, EUA, 1996). When possible, we used one algorithm (logistic dose response) as our equation of choice.

Small-animal model of infection with ETEC. All mouse intestinal assay procedures were approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee. The diet-restricted, antibiotic-treated mouse model was used to evaluate the effect of the adenyl cyclase inhibitors on various ETEC strains. We selected the mouse intestinal model to study enteropathogenicity of *E. coli* strains (6, 24) that our lab has modified from the work of Allen et al. (1). Briefly, we used female ICR mice of 20 to 25 g (Charles River Laboratories) that were housed in the pathogen-free animal facility at UTMB upon arrival for 72 h prior to experiments. The animals' food was restricted and altogether removed 12 h prior to inoculation. All animals received streptomycin (5 g/liter in drinking water supplemented with 7% fructose) for 48 h prior to oral inoculation with ETEC (32). Four h prior to inoculation, the fructose-treated water was replaced with unsweetened, sterile water. Cimetidine (50 mg/kg) was injected into all mice 1 to 3 h prior to oral inoculation with ETEC strains (27). Groups of 6 to 10 mice were orally inoculated with a suspension of ETEC bacteria in the presence or absence of the inhibitors (0.1 mM or 1 mM DC5 or PGE₂-imidazole) in a final volume of 0.4 ml delivered by gavage (20-gauge needle). In some cases, mice were dosed with various concentrations of the compounds (200 µl) via the intraperitoneal route at 12-h intervals (12, 24, 36, 48, and 60 h). The animals were maintained for 24 or 72 h depending

on the experimental protocol, but 12 h prior to euthanasia, food was withdrawn. To collect the fluid accumulated in the small intestine, ligatures of 2-0 Vicryl suture were created in each mouse and placed at the junctions of the pylorus and duodenum, as well as the ileum and cecum. After euthanasia, the entire small intestine of each mouse was excised and weighed. To collect total bacteria, 200 µl of PBS was then injected into one end of the ligated intestine, the other end was cut, and the total content, containing bacteria, was then harvested from the small intestine and collected in microcentrifuge tubes. Serial dilutions of the intestinal content were plated in duplicate on MacConkey agar plates or LB-streptomycin agar plates and incubated at 37°C overnight. The quantification of the organisms is reported as CFU/intestinal segment. Fluid accumulation was analyzed by a two-tailed Student's *t* test for independent samples or by Dunnett's multiple group comparison test.

Hematology. In order to perform a cursory examination of DC5's toxicity, various blood markers were examined. CD-1 mice were anesthetized with isoflurane, and a cardiac puncture was performed to remove roughly 1 ml of blood. One half of the blood volume was placed in a K2EDTA tube (BD Microtainer) to determine leukocyte counts via a Hemavet 950FS. The other half of the blood was placed in a Z tube (BD Microtainer) and allowed to coagulate at room temperature for 4 h, at which time the samples were centrifuged and serum was extracted. The serum was analyzed in a Vegasys blood chemistry analyzer for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine phosphokinase (CPK), common indicators of toxicity.

Preparation of samples for electron microscopy. Segments of mouse intestinal tissue infected with the wild-type ETEC strain were collected from ileal segments, washed gently with PBS, and fixed in a mixture of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, and 0.03% CaCl₂ in 0.05 M cacodylate buffer (pH 7.2). After being washed in 0.1 M cacodylate buffer, the tissues were processed further by postfixing in 1% OsO₄ in the same buffer, stained *en bloc* in 1% uranyl acetate in 0.1 M maleate buffer (pH 5.2), dehydrated in ethanol, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Specimens were examined in a Philips 201 electron microscope. To eliminate bias in our electron microscopy (EM) analysis, we took samples from representative mice and embedded 10 pieces of tissue from each sample, randomly selecting pieces for cutting and further processing. There was one observer examining the sections blindly and taking pictures randomly of each sample.

RNA extraction and preparation for RT-PCR. Animals infected with ETEC or ETEC plus DC5 were euthanized, and 5- to 10-cm sections of the small intestine were removed and immediately flushed with PBS to eliminate nonadherent bacteria. The intestinal segments were macerated and resuspended in 10-fold volumes of RNeasy Protect (Qiagen). Samples stabilized with RNAlater (Invitrogen) were snap-frozen with liquid nitrogen, stored at -80°C, and thawed on ice prior to bacterial extraction. Samples were treated with 1% Triton X-100 and vortexed to remove adherent bacteria. For purification, a 5-fold dilution of the RNAlater in distilled water was performed and connective tissue and cellular debris were removed by centrifugation at 1,000 × *g* for 10 min at 4°C. Supernatant was collected and bacteria were pelleted by centrifugation at 12,000 × *g* for 20 min at 4°C. Bacterial RNA was extracted using a Qiagen RNeasy Protect Bacteria mini kit. Quality of RNA was assessed by determining the ratio of optical density at 260 nm to the optical density at 280 nm (OD_{260/280} ratio) and by visualization following agarose gel electrophoresis and ethidium bromide staining. The cDNA was synthesized using the SuperScript first-strand synthesis system for reverse transcriptase PCR (RT-PCR) (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA was utilized for regular PCR with gene-specific primers to amplify *elt* (heat-labile enterotoxin of ETEC) (forward 5'-GGC GAC AGA TTA TAC CG TGC-3' and reverse 5'-CGG TCT CTA TAT TCC CTG TT-3') and using PCR conditions previously described by our group (5). A positive control with genomic DNA and a negative control with no reverse transcriptase added were also used in the assay.

Adhesion assays with different strains in the presence or absence of DC5. Adherence of bacterial strains to cultured HeLa cells was evaluated as previously described (5). DMEM with 10% (vol/vol) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100,000 IU/liter), and streptomycin (100 mg/liter) was used to grow HeLa cells prior to bacterial infection. Bacteria were grown statically in LB broth overnight at 37°C and inoculated at a multiplicity of infection of approximately 10:1 onto semiconfluent cultured epithelial cell monolayers grown in 24-well microtiter plates. Before use, the cells were washed with sterile phosphate-buffered saline (PBS, pH 7.4) and replenished with DMEM. For *in vitro* experiments assaying the effect of exogenous LT, HeLa cells received 100 ng/ml LT in the presence or absence of DC5 (0.1 mM) compound prior to ETEC infection, and they were incubated for 3 h at 37°C and 5% CO₂. To quantify bacterial adherence, the cells were washed two times with PBS and

FIG. 1. *In vitro* cell-based cAMP assay. (A) Bioassay with different concentrations of PGE₂-imidazole and DC5 demonstrating that these compounds inhibited cAMP production induced by edema toxin (EdTx) on RAW 264.7 cells, with IC₅₀s of 14.1 and 8.99, respectively. Controls were medium alone or with the addition of 100 μ M each individual compound tested (protective antigen [PA] or edema factor [EF]). (B) DC5 effect on cAMP production by cells treated with CT (equivalent to LT, IC₅₀ of 2.04).

bacteria were recovered with 0.1% Triton X-100 in PBS buffer and plated on Luria agar plates.

RESULTS

DC5 prevents accumulation of cAMP by cultured cells. Recently, a structure-based procedure, involving compound library screening using a fragment-based 3D-pharmacophore, was used to identify compounds that should bind to the active site of edema factor, an adenyl cyclase toxin (3). A small

number of diverse compounds were identified that reversed cAMP secretion induced by these toxins and had no apparent toxicity on cultured cells (3, 4). One of the leading compounds for blocking cAMP accumulation by cultured cells, named DC5 {3-([9-oxo-9H-fluorene-1-carbonyl]-amino)-benzoic acid}, was selected for our analysis. The inhibitory effect of the DC5 compound on preventing accumulation of cAMP was compared to that of a previously known inhibitor, PGE₂-imidazole (21). Data illustrated in Fig. 1A demonstrated that DC5 had

FIG. 2. Bacterial growth in LB medium in the presence or absence of DC5. The different bacterial cultures were monitored spectrophotometrically for a period of 6 h. (A) ETEC H10407, EPEC E2348/69, EHEC 86-24. (B) ETEC DEC7A, EPEC E2348/69, *S. Typhimurium*, EAEC O42.

more activity than PGE₂-imidazole inhibiting the accumulation of cAMP caused by edema toxin (EDTx) treatment, as shown by the lower IC₅₀ (IC₅₀ = 8.99 μ M for DC5 [R^2 = 0.986] compared to IC₅₀ = 14.10 μ M for PGE₂-imidazole [R^2 = 0.992]). Similarly, DC5 was effective in reducing the accumulation of cAMP due to treatment of RAW 264.7 cells with cholera toxin (Fig. 1B; CT and LT share an identical mode of action), as indicated by the lower IC₅₀ (IC₅₀ = 2.04 μ M [R^2 = 0.987]). These data suggest that DC5 is an effective inhibitor of toxin activity *in vitro*.

Bacterial growth kinetics in the presence or absence of DC5.

Next, we determined whether DC5 had any effect on growth of bacterial strains producing the LT toxin or other enteric organisms (Fig. 2 and data described below). First, we performed a series of *in vitro* growth studies to examine whether DC5 had any antibacterial effect on cultures of our prototype ETEC H10407 grown in Luria-Bertani (LB) broth in the presence or absence of 0.1 mM DC5. Growth of ETEC was monitored by measuring the OD₆₀₀ at 1-h intervals for 6 h, as well as quantification by serial dilution and plating bacteria at 0 h and 6 h. Our data showed that DC5 had no effect on the growth of ETEC (the numbers of CFU recovered on the plates matched the OD₆₀₀ data), which suggested that the effects observed during intestinal colonization of our animal model (see sections below) are not linked to inhibition of the growth or killing of the bacteria by the DC5 treatment (Fig. 2A). Based on these results and previous studies demonstrating that DC5 inhibits the activity of CT in the murine ileal loop model (data not

FIG. 3. Establishing the optimal DC5 dose. (A) Increase in fluid (estimated by weight of the intestine) was reduced in the presence of DC5. *P* values (*t* test): *, <0.05 (comparing DMSO plus PBS control with ETEC treatment); **, <0.05 (ETEC treated with ETEC plus 1 mM DC5). (B) The DC5 treatment had an impact on the amount of bacteria (ETEC) recovered from the intestinal lumen at 72 h, particularly at 0.1 mM DC5. *P* values (*t* test): *, <0.01 (comparing ETEC treatment with ETEC plus 0.1 mM DC5 or ETEC plus 1 mM DC5). Each group included eight mice.

shown), we tested whether this compound had activity against LT-producing ETEC.

Establishing the optimal DC5 dose. The initial studies were used to establish an optimal DC5 concentration in our murine model. CD-1 mice (n = 8/group) were divided into four groups on the basis of dose received: (i) DMSO (at the concentration used to solubilize DC5) plus PBS; (ii) ETEC (1×10^8) bacteria; (iii) ETEC bacteria plus 1 mM DC5; (iv) ETEC bacteria plus 0.1 mM DC5. The DC5 intraperitoneal (i.p.) boosters were administered at 12, 24, 36, 48, and 60 h prior to euthanasia at 72 h. At that time point, intestine weights and CFU counts were established; as shown in Fig. 3, DC5 effectively reduced the intestinal fluid accumulation after experimental ETEC infection, and although the difference is not dramatic, it was statistically significant (Fig. 3A; P = 0.02). Statistically significant differences were also observed between results for mice infected with ETEC and those receiving the vehicle (PBS plus DMSO) control (P = 0.03) and between results for animals treated with PBS plus DMSO control and ETEC-plus-DC5-treated animals (P = 0.003). To our surprise, the number of bacteria recovered from the intestine after infection was

FIG. 4. Comparing the effects of DC5 and PGE₂-imidazole. (A) Reduction in the weight of the intestine (indicative of fluid accumulation) was observed in DC5- and PGE₂-imidazole-treated mice. *P* values (*t* test): *, <0.05 (comparing ETEC treatment with ETEC plus 0.1 mM DC5). (B) Both compounds had a significant effect on bacterial recovery from the intestinal lumen at 72 h postinfection. *P* values (*t* test): *, <0.01 (comparing ETEC treatment with ETEC plus 0.1 mM DC5 or ETEC plus 0.1 mM PGE₂-imidazole). Each group included eight mice.

significantly reduced in the infected animals treated with DC5 compared with results for controls (Fig. 3B). Because the differences between 0.1 and 1 mM DC5 were not significant, we decided to use the lower DC5 concentration (0.1 mM in the 0.4-ml dose) for subsequent experiments.

Comparing the effects of DC5 and PGE₂-imidazole in the murine model of ETEC infection. Because our data *in vitro* indicated that DC5 has more activity than PGE₂-imidazole, we then determined whether dosing mice with DC5 was more effective than, or as effective as, treating the animals with PGE₂-imidazole at the same concentration. Similar to Fig. 3, animals were divided into groups and received ETEC (1×10^8) with or without DC5 (0.1 mM) or PGE₂-imidazole (0.1 mM). DC5 or PGE₂-imidazole i.p. boosters were administered at 12, 24, 36, 48, and 60 h prior to sacrifice of the animals at 72 h. As shown in Fig. 4, DC5 was slightly more effective in reducing the fluid accumulation in the intestine than PGE₂-imidazole (Fig. 4A). Most importantly, both compounds had a direct effect on the number of bacteria recovered from the intestinal lumen (Fig. 4B). These results strongly suggested that the ability of the ETEC bacteria to colonize and persist in the intestine was associated with the presence of the LT toxin, an effect that was diminished upon the addition of PGE₂-imidazole or DC5.

We then sought to perform an ultrastructural analysis of the infected intestinal tissues by electron microscopy to determine

whether the inhibitors had a detrimental impact on the epithelia while diminishing the effect of the LT toxin. As shown in Fig. 5, ETEC-only infected tissues showed destruction of the microvilli of the cells, with areas of cell death detected in the sections analyzed (Fig. 5A to C). The integrity of the tissue was consistently different from that observed in control experiments where the intestine was infected with a nonpathogenic *E. coli* strain (data not shown). When the ETEC-infected animals were treated with PGE₂-imidazole, we noticed changes in the structural integrity of the intestinal epithelial barrier, however, and upon close examination, we noticed that the microvilli of the cells looked slightly damaged (small areas of microvillus destruction), but no evidence of cell death was found (Fig. 5D to F). In contrast, the integrity of the intestinal epithelial barrier was fully maintained in those CD1 mice infected with ETEC but receiving DC5 (Fig. 5G to I). We then evaluated whether treatment with DC5 caused damage to other organs (spleen, liver, or the rest of the intestine) or affected blood cell counts or liver enzymes. Our analysis indicated that the number of leukocytes in circulation was not affected in the animals treated with DC5. Further, no differences in the weight or the ultrastructure of liver, spleen, and intestine were observed compared to those of control mice (data not shown). Our results suggest that the microvilli on the epithelial cells remained intact upon treatment with the two compounds, with no evidence of cellular death, and that DC5 treatment was more effective than PGE₂-imidazole in preventing intestinal damage due to ETEC infection.

To confirm that the effect in the microvilli was due to the interaction of the bacteria with the intestinal mucosa, resulting in production of toxins, and not just to a lack of interaction with the intestinal cells, we performed an RT-PCR analysis of bacteria attached to the intestinal mucosa ($n = 6$ per group). Our result indicated that a positive PCR was obtained from all mice infected with ETEC but only in half of mice receiving ETEC plus DC5. This result suggested that the presence of DC5 inhibits LT toxin activity, affecting the ability of ETEC to colonize the intestine (data not shown). Subsequent experiments were designed to determine whether the reduction of fluid accumulation observed at 72 h postinfection in animals treated with DC5 was achieved early during infection. In this case, animals infected with ETEC (1×10^8) and treated with DC5 (0.1 mM) at time zero received DC5 boosters administered i.p. at 12, 24, 36, 48, and 60 h. Groups of animals were euthanized at 24, 48, and 72 h. We observed a transient increase in fluid accumulation at 24 h in animals treated with DC5 compared to accumulation in ETEC-only infected animals, but this fluid accumulation progressively decreased over a 24- to 72-h time period (data not shown). Interestingly, we still observed 3-log differences in the number of bacteria recovered from animals euthanized at 72 h ($P < 0.01$ in animals treated with ETEC plus DC5 versus those treated with ETEC alone).

Determining whether DC5 route of administration had an effect on bacterial recovery. To evaluate whether the route of administration of DC5 had an impact on bacterial recovery, ETEC-infected animals received DC5 by the oral or the i.p. routes at time zero and DC5 boosters were administered via i.p. or orally at 12, 24, 36, 48, and 60 h postinfection. Animals were euthanized at different time points. Physical signs of dam-

FIG. 5. EM images of infected intestine. Ultrastructural studies of the intestine infected with ETEC only (A to C), ETEC treated with PGE_2 -imidazole (D to F), and ETEC with DC5 (G to I). ETEC-infected intestines demonstrated destruction of the microvilli and cell death, while integrity of the microvilli in the compound-treated animals remained intact. Control tissue (noninfected) was used for comparison (not shown). Magnification: panels A, D, and G, $\times 16,000$; panels B, E, and H, $\times 32,000$; panels C, F, and I, $\times 64,000$.

age (inflammation, fluid accumulation, bleeding, etc.) were evaluated, along with determination of the bacterial counts. No signs of bleeding or inflammation were observed in any of the animals receiving DC5, but fluid accumulation was observed at 24 h (ETEC plus DC5 oral dosing). Bacteria were recovered in animals infected with ETEC alone, and a significant reduction ($P < 0.01$) in the ETEC bacterial counts was observed in mice treated with DC5, especially at 72 h postinfection, regardless of the route of administration (Fig. 6). No bacteria were recovered in animals receiving only DC5 (data not shown).

Bacterial adhesion to cultured epithelial cells. First, we determined whether DC5 had any antibacterial effect (growth defect) on cultures of other enteric pathogens (including isolates of pathogenic *E. coli* [ETEC, EHEC, EPEC, and EAEC] and *S. Typhimurium*) grown in Luria-Bertani (LB) broth in the presence or absence of 0.1 mM DC5 (Fig. 2). Similar to our

results with the prototype ETEC H10407, we showed that DC5 had no effect on the growth of any of the strains tested, which further supports that the effects observed during ETEC intestinal colonization are not the result of inhibition of the growth or killing of the bacteria by the DC5 treatment (Fig. 2). Because we did not observe any difference in growth rate of the bacteria treated with DC5, we next determined whether DC5 affected bacterial adhesion to epithelial cells. Semiconfluent cultured HeLa cell monolayers (20) were infected (multiplicity of infection [MOI] of approximately 10:1) with the pathogenic *E. coli* (ETEC [two strains], EPEC, EAEC, and EHEC) as well as *S. flexneri*, *S. Typhimurium*, and *V. cholerae*, in the presence or absence of DC5. ETEC strains produced LT; *V. cholerae* produced CT, and the other pathogens produced a variety of secreted/translocated toxins. As shown in Fig. 7, we found a reduction in adherence with the two ETEC strains treated with

FIG. 6. Comparing effects of DC5 routes of administration on bacterial survival. ETEC-infected animals received DC5 treatment using the oral or the intraperitoneal routes at 24, 48, and 72 h postinfection, and the CFU in the intestine were calculated. *P* values (*t* test): *, <0.01 (comparing ETEC treatment with ETEC plus 0.1 mM DC5 or ETEC plus 0.1 mM PGE₂-imidazole). *P* values (*t* test): *, <0.01 (comparing treatments at 72 h, ETEC treatment versus ETEC plus 0.1 mM DC5 IP or ETEC plus 0.1 mM DC5 orally administered). Each group included eight mice.

DC5 ($P < 0.05$), compared to that with the cells infected with only the ETEC strains. Interestingly, we also observed reduction in adherence in EAEC, EPEC, and EHEC, strains that do not produce LT toxin. In contrast, a slight increase in adherence was observed in *V. cholerae*, *S. enterica*, and *S. flexneri* (not statistically significant). It was surprising to observe an increase in adherence of *V. cholerae* in the presence of DC5, which suggests that adherence of this pathogen might be independent of the production of the CT toxin.

To determine whether the LT toxin was playing a selective role in adherence for the ETEC strains, we performed additional experiments where exogenous toxin was added to the cultured media in the presence or absence of DC5 and prior to the pathogens' infections (Fig. 7). We observed that the adherence of the majority of the strains was not increased in the presence of the LT toxin and in contrast, several strains displayed a reduction in adherence compared to that of their respective reference wild-type strains. Due to the presence of the exogenous LT toxin, DC5 effect on adherence was eliminated, perhaps due to binding of the compound to the toxin found in solution. Overall, our results suggested that DC5 is a promising compound that can be used to treat infections caused by LT-positive bacteria and, potentially, other toxin-producing isolates, and they supported the importance of adenyl cyclase LT toxin as a mediator of binding of ETEC strains to epithelial cells.

DISCUSSION

The data derived from this study had two major implications. First, a best-fit *in silico*-derived compound based on the active site binding of adenyl cyclase toxins, such as LT, was demonstrated to prevent accumulation of cAMP by cultured cells. Second, our murine model data confirmed previous reports that LT provides a colonization advantage for ETEC *in vivo* (16) and that inhibition of the toxin activity had a direct inhib-

itory effect on the ability of this organism to adhere to host cells.

It has been proposed that diarrhea caused by enterotoxigenic bacteria depends on the activity of endogenous host mechanisms; however, our understanding is still relatively poor regarding the exact mechanisms involved. It is evident that enterotoxins like LT play an important role, but the enteric nervous system and inflammatory cells are also involved. Indeed, intestinal cells respond to the infection by switching on innate defense mechanisms (7), which will eventually determine the outcome of the disease. The concept that toxins condition the enterocyte for enhanced colonization was observed during work with the CT toxin, where it was concluded that this toxin contributes significantly to mucosal colonization by *V. cholerae* and that this effect is not due to an interaction of the CT B subunit with its mucosal receptor (23). In animal studies using *V. cholerae*, strains lacking either *ctxA* or both *ctxA* and *ctxB* (encoding the A and B subunits of CT, respectively) and the fully virulent *V. cholerae* expressing CT demonstrated that the bacteria expressing the intact CT were better colonizers of the gut in the infected animal (22), and toxin-negative strains could be made to colonize the gut similarly by preconditioning the gut through preexposure to CT (23). In ETEC, a similar mechanism of LT-mediated colonization has been proposed (1, 8, 16, 23). In these studies, it has been shown that LT promotes the adherence of ETEC to intestinal epithelial cells *in vitro* and *in vivo*. Similar to the *V. cholerae* experiments, deletions of *eltA* or both *eltA* and *eltB* (encoding A and B subunits of LT) in ETEC reduced adherence to intestinal epithelial cells and administration of LT was sufficient to increase adherence of the ETEC *eltAB* double mutant (16). These data also suggested that exposure of the epithelial cells to the toxin will result in a host response that will enhance binding of the ETEC bacteria to the cell. Thus, it is logical to think that interruption of the host response with a drug would limit fluid loss resulting from decreased colonization and toxin production. Therefore, any compound that can interrupt the effect of the toxin on the intestinal cells would be a logical therapeutic approach to prevent diarrheal disease.

Results presented in our study suggest that the newly identified adenyl cyclase inhibitor DC5 could prove to be effective in the treatment of enterotoxigenic diarrhea. We demonstrate that DC5 prevents secretion of cAMP by cultured cells and this compound is more active than PGE₂-imidazole. The inhibitory effect was dose dependent, and the IC₅₀s were relatively small. It has been well established that LT toxin increases intracellular cAMP levels by activating adenylate cyclase through the GTP-dependent ADP ribosylation of Gs α and that the channel responsible for enterotoxin-induced Cl⁻ secretion is the CFTR Cl⁻ channel (reviewed in reference 8). We ruled out the possibility that receptor antagonism and G protein inhibition could account for the inhibition, because DC5 was specifically selected to bind to the active site of bacterial toxins and, therefore, not interfering with the function of cellular adenyl cyclases (3, 15). While we cannot completely exclude that DC5 could mediate its effect by modulation of endogenous host adenylate cyclases, it was previously reported (21) that in the case of PGE₂-imidazole, the incorporation of the imidazole moiety inactivates the native stimulatory effect of PGE₂ on ion transport (PGs are well known as inflammatory mediators and

FIG. 7. Effect of DC5 and LT on bacterial adhesion to HeLa cells. Bacteria were inoculated at a multiplicity of infection of 10:1 onto semiconfluent cultured epithelial cell monolayers in the presence or absence of DC5 or exogenous LT toxin. Bacteria and cells were incubated for 3 h, and the adherent bacteria were quantified by serial dilutions. (A) ETEC H10407, EAEC O42, *V. cholerae* 596B; (B) EPEC E2348/69, EHEC O157:H7, ETEC DEC7A; (C) *S. enterica* serovar Typhimurium, *S. flexneri*. Each panel represents duplicate independent experiments performed in triplicate. *, $P = 0.05$; **, $P = 0.01$ (t test; comparing treated samples to their corresponding wild-type strains).

secretagogues in the gastrointestinal tract). Therefore, it is plausible to propose that DC5 interferes with the action of LT-induced fluid accumulation without affecting endogenous adenylate cyclases. Further, inhibition of LT toxic action could prevent an overall increase in localized, secreted cAMP, which could, for example, affect quorum sensing signaling (26) and eventually have an impact in ETEC colonization.

The idea that DC5 might show promise in reducing the adherence of other pathogenic *E. coli*, *Salmonella*, *Shigella*, and *V. cholerae* strains to epithelial cells and, if effective, might show promise as a compound reducing diarrheal symptoms during infection across species was evaluated *in vitro*. Our results showed that this compound was effective only against LT-producing strains and no other enteric pathogens. We were surprised not to see a reduction in adherence capabilities of *V. cholerae* in the presence of DC5, but it is plausible to speculate that differences in secretion of these toxins (CT versus LT) upon contact with the cells might be associated with this difference. It has been shown that one major difference between

the CT and LT lies in the fact that although CT is secreted from the cell, LT reportedly remains periplasmic (9–12), and some other studies have also found LT to be associated with membranes extracellularly (13, 14, 33, 35). Our *in vitro* assays support this hypothesis because addition of exogenous LT to the prototype ETEC strain abolished the reduction in adherence observed in the presence of DC5. Despite the equivalent activities that CT and LT exhibit in bioassays, disease caused by ETEC is usually much less severe than that caused by *V. cholerae* (19). This finding suggests that the difference between *V. cholerae* and ETEC virulence may depend on the efficiency of toxin secretion and the delivery mechanism (30). Despite this discrepancy, our own studies using the ligated loop murine model of infection with CT demonstrated that DC5 inhibited fluid accumulation in the intestinal lumen of infected mice (data not shown). Therefore, DC5 might be an effective compound to treat enterotoxigenic diarrhea caused by LT- or CT-producing bacteria without affecting the integrity of the intestinal epithelia.

The use of a relatively low and therapeutic dose of DC5 (ca. 15 μ g/mouse treatment) and the reduction of gut barrier destruction in the infection model suggest that this compound might help in the stabilization of the gut epithelial barrier function by blocking the activity of LT toxin produced by ETEC bacteria directly inoculated into the gastrointestinal tract of mice. Interestingly, the effect against bacterial colonization was not due to an antimicrobial activity exerted by DC5. Recently, a study developed innovative therapeutic approaches for acute diarrhea caused by ETEC, and this was based on an inhibitor of stimulated cyclic nucleotide synthesis (17). The leading compound of the study, BPIPP [5-(3-bromophenyl)-1,3-dimethyl-5,11-ihydro-1*H*-indeno(2',1':5,6)pyrido(2,3-*d*)pyrimidine-2,4,6-trione], inhibited the stimulation of guanylyl cyclases (e.g., heat-stable toxin A [STa] of ETEC) in various cells, and suppressed stimulation of adenylyl cyclases. Further, this study showed that BPIPP concentration-dependently suppressed STa-stimulated water and electrolyte secretion in an *in vivo* rabbit intestinal loop model and proposed that the compound can potentially be used for therapy of toxin-induced secretory diarrhea (17). Their compound and ours are effective in treating enterotoxigenic diarrhea; although their different molecular mechanisms of action are not completely elucidated, it is plausible to suggest that the combination of these compounds could suppress the formation of cyclic nucleotides, cAMP and cGMP, and their diarrheagenic effects.

In conclusion, this study demonstrated that the function of LT in ETEC pathogenesis includes its role as a key stimulator of cAMP-mediated fluid loss from the cells, as well as its role in bacterial adherence to epithelial cells. DC5 is a promising lead compound for treatment of diarrhea caused by ETEC and, perhaps, other adenylyl cyclase toxin-producing isolates. However, further studies are needed to define the DC5-related inhibitory mechanisms preventing gut barrier LT-mediated destruction.

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