Cyclooxygenase (Cox)–2 expression and inhibition were investigated in a rabbit ileal loop model of Clostridium difficile colitis and diarrhea. Intestinal tissue stimulated with C. difficile toxin A showed up-regulation of Cox-2 expression in lamina propria macrophages and elevated prostaglandin levels. Toxin A–stimulated loops exhibited severe inflammation and increased secretory volume. Celecoxib, a specific Cox-2 inhibitor, significantly reduced toxin A–induced prostaglandin production. Furthermore, celecoxib (≥0.02 mg/mL) blocked both histologic damage (mean histologic grade, 1.25 vs. 3.44 in rabbits receiving toxin A alone; \(P < .0005\)) and secretion (volume:length ratio, 0.18 vs. 0.72 in those receiving toxin A alone; \(P = .002\)) in toxin A–stimulated loops in a dose-related manner. Thus, toxin A induced expression of Cox-2 in the host, and prostaglandins produced through Cox-2 were involved in the mediation of the increased secretion of electrolytes and water and the inflammatory response induced by toxin A.

Prostaglandin E\(_2\) (PGE\(_2\)), an agent that increases salt and water secretion in the intestine, induces cAMP-mediated chloride secretion and inhibits neutral sodium chloride and water absorption [5]. PGE\(_2\) is a metabolite of arachidonic acid and is synthesized through the cyclooxygenase (Cox) pathway. There are 2 isoforms of this enzyme: Cox-1, which is constitutively expressed in crypt epithelial cells, and Cox-2, which can be induced in a variety of cell types, including epithelial cells, macrophages, and fibroblasts. Cox-2 is induced by proinflammatory cytokines, lipopolysaccharide, and infectious agents [6].

Some other infectious colitides are associated with the induction of a number of proteins in affected epithelial cells. For example, invasion of epithelial cells with Salmonella organisms induce the expression of Cox-2 [7]. PGE\(_2\) produced through Cox-2 in Salmonella-infected epithelial cells contributes to apical chloride secretion and thus to the development of diarrhea. In this study, we sought to determine whether toxin A induces the expression of Cox-2 in the rabbit intestinal model of colitis and secretion and to determine the contribution of Cox-2–derived prostaglandins to the host inflammatory and secretory responses to toxin A in the intestine.

**Methods**

**Immunohistochemistry.** For immunohistochemical localization of rabbit Cox-2, deparaffinized sections of Bouin’s-fixed ileal loop tissue were incubated overnight at 4°C with a 1:500 dilution of mouse monoclonal Cox-2 antibody (Transduction Laboratories) [8]. Sections also were incubated with normal mouse serum or without primary antibody, to serve as negative controls. After overnight incubation, sections were treated with 3% hydrogen peroxide to quench exogenous peroxidase activity and were incubated with donkey anti–mouse IgG conjugated to biotin (Jackson ImmunoResearch Laboratories).
Ligated rabbit ileal loops. Six 2-kg New Zealand White rabbits were fasted overnight. After anesthesia with ketamine and xylazine (60–80 and 5–10 mg/kg intramuscularly, respectively), each rabbit was shaved, and a midline incision was made. The distal 40–60 cm of the ileum was exposed and was flushed with PBS. In total, 8–11 4-cm ileal segments were doubly ligated at each end. Three rabbits were control animals, and 3 were treated. Control loops (n = 14) were injected intraluminally with PBS only or with PBS plus 20 μg of C. difficile toxIN A (Techlab), each with a total volume of 1 mL/loop. Treatment loops (n = 14) were injected with celecoxib, a selective inhibitor of Cox-2 [9] at doses of 5, 2, 0.2, or 0.02 mg/mL immediately before administration of either PBS or toxin A. The ileal loops were returned to the abdominal cavity, and the incision was sutured closed.

After incubation for 5 h, the rabbits were euthanized, and the intestinal loops were immediately removed. The length of each segment was remeasured, and the intraluminal fluid was extracted. We recorded the character and volume of the fluid and calculated the volume-to-length (V:L) ratio in milliliters per centimeter for each loop.

A piece of the intestinal tissue was fixed in 10% formalin and was submitted for histopathologic study. The tissues were stained with hematoxylin-eosin (HE) and were read randomly by 2 investigators who were unaware of the treatments used. A grading scale (not based on the basis of degree of mucosal disruption, cellularity, and vascular congestion, 0 (intact mucosa, normal cellularity, and no congestion) to 4 (denuded mucosa, intense inflammation, and severe congestion).

Ileal tissues from another 2 rabbits were used for immunohistochemistry. Intestinal loops from the control and treated rabbit were injected with PBS only and C. difficile toxin A and PBS, respectively. These loops were incubated for 2 h to permit adequate visualization of the intestinal mucosa for Cox-2 expression.

Results

Cox-2 expression is enhanced in C. difficile toxin A–stimulated ileum. Immunohistochemistry in the normal rabbit ileum revealed no staining for Cox-2 (figure 1A). Ileum treated with toxin A showed Cox-2 staining in a population of lamina propria cells. These rounded cells were scattered throughout the lamina propria but were more prominent in the villi. The shape and distribution of these cells were most consistent with a subpopulation of macrophages (figure 1B and 1C). There was also staining of linear structures in the villi that appeared to be blood vessels (figure 1C), as well as staining of endothelial cells in veins in the submucosa (figure 1D).

Elevated PGE2 production in C. difficile toxin A–stimulated ileum is blocked by Cox-2 inhibition. PGE2 concentration was increased ∼20-fold in toxin-treated ileum. The control tissues (n = 3) had a mean ± SEM PGE2 concentration of 7.24 ± 1.78 pg/mg tissue, whereas C. difficile toxin A–stimulated loops (n = 4) had a mean PGE2 concentration of 139.25 ± 24.58 pg/mg tissue (P = .01). Treatment (n = 3) with celecoxib (2 mg/mL) before toxin A decreased PGE2 production to 57.83 ± 19.63 pg/mg tissue, which is a 62% decrease versus that in loops receiving toxin A alone; (P < .05).

Cox-2 inhibition blocks C. difficile toxin A–induced secretion. Toxin A loops pretreated with celecoxib (all doses; n = 10) had a mean ± SEM V:L ratio of 0.18 ± 0.06, which is a 75% decrease versus that in loops receiving toxin A alone (n = 8), which had a mean V:L ratio of 0.72 ± 0.12 (P = .002; figure 2). Inhibition of secretion followed a concentration-dependent response curve. Impressive inhibition was observed at all celecoxib doses tested, with dose-dependent reductions in secretion of 100%, 87%, 90%, and 48% at 5, 2, 0.2, and 0.02 mg/mL/loop, respectively (P = .009, ANOVA).

Cox-2 inhibition blocks mucosal injury and inflammation. HE-stained sections of ileal loops treated with toxin A plus celecoxib had substantially less mucosal disruption, inflammatory cellular infiltration, and vascular congestion than loops treated with toxin A alone (figure 2). The mean histologic grade of the loops treated with toxin A alone was 3.44 versus 1.25 for loops also treated with celecoxib (n = 10; P < .0005). Significant inhibition of mucosal disruption and inflammation was observed across all doses (data not shown). No difference was observed between loops treated with celecoxib and PBS versus PBS alone.

Discussion

In this study, we sought to determine whether administration of toxin A, like infection with invasive bacteria [7], can induce epithelial cell Cox-2 expression and whether that expression contributes to toxin A–induced fluid secretion. We found that the administration of toxin A induces Cox-2 expression not in epithelial cells but in a lamina propria cell population that appeared to be macrophages. Toxin A has a direct effect on epithelial cells but in a lamina propria cell population that appears to be macrophages. Toxin A induces the production of interleukin (IL)–1, IL-6, and IL-8 and tumor necrosis factor (TNF)–α in peripheral blood monocytes in vitro [11, 12]. Not only does this demonstrate a direct response of monocytes to toxin A but also indicates the existence of a common intra-
cellular signaling pathway, NF-κB, in the induction of IL-1, IL-6, and IL-8, TNF-α, and Cox-2 [13]. In many systems, agents that induce the expression of IL-1, IL-6, and IL-8 and TNF-α also induce expression of Cox-2 [7].

Both toxin A and toxin B induce the glucosylation of a threonine in the small GTP binding protein, rho [3, 4]. Microinjection of glucosylated rho proteins into monolayers of PtK2 cells results in disaggregation of the actin filaments, indicating a dominant-negative activity of glucosylated rho proteins. Activation of rho proteins induce Cox-2 transcription through a pathway dissociated from rho-dependent actin polymerization [14]. This suggests that glucosylation of rho proteins also could play a role in the induction of Cox-2 expression in toxin A–stimulated intestinal tissues.

Toxin A induces the production of PGE₂ through Cox-2. This probably represents increased PGE₂ production by macrophages as a result of increased Cox-2 levels. Although it is possible that other cell types also contribute to PGE₂ production, there is no suggestion of Cox-2 expression in other cell types (except for endothelial cells) by immunohistochemistry. PGE₂ induces cAMP-mediated apical chloride secretion and inhibits electroneutral sodium chloride and water absorption in enterocytes. Toxin A induces increased water and electrolyte secretion into the lumen, and this effect is largely blocked by the administration of a Cox-2 inhibitor. The likely mechanism is that PGE₂ produced in the lamina propria induces enterocyte chloride secretion. Infection of epithelial cell lines with Salmonella organisms induces the expression of Cox-2, and PGE₂ produced through Cox-2 in these cells induces apical chloride secretion [7].

Treatment of ileal loops with toxin A also induces inflammation with neutrophil infiltration, epithelial disruption, and
vascular congestion. Administration of a Cox-2 inhibitor reduced the inflammatory response and epithelial injury. The mechanisms by which prostaglandins produced through Cox-2 promote these inflammatory responses are not clear, but prostaglandins, like toxin A, promote the production of IL-8, an important neutrophil chemotactic agent [15].

In this study, we demonstrate that injection of a rabbit ileal loop with toxin A results in the induction of Cox-2 expression in the lamina propria and an increase in intestinal prostaglandin levels. Moreover, the prostaglandins produced through Cox-2 mediate, in part, the increased water and electrolyte secretion and the inflammatory response induced by toxin A. These findings with immediate pretreatment in a rabbit ileal loop model suggest that a selective Cox-2 inhibitor might offer a nonantimicrobial alternative to current antibiotic therapy, to reduce the diarrhea and the inflammatory response in C. difficile colitis or pseudomembranous colitis; however, effects with established disease must be examined. The findings presented here, combined with difficulties with current antibiotic therapy for C. difficile colitis, including predisposition to relapse and the emergence of resistant organisms such as vancomycin-resistant enterococci, suggest that further study of potential pharmacologic means to block the effects of C. difficile toxins are clearly warranted.

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References