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.

*C. difficile* is the major cause of nosocomial diarrhea, accounting for up to 25% of all antibiotic-associated diarrhea and for most cases of pseudomembranous colitis. *C. difficile* produces intestinal damage by releasing 2 exotoxins, toxins A and B. Toxin A, a 308-kDa heat-labile, protease-sensitive protein, elicits secretion of fluid in rabbit ileal loops [1]. The fluid released is an inflammatory exudate containing lymphocytes, neutrophils, and serum proteins. The induction of fluid secretion and inflammation by toxin A is thought to be the major mechanism of diarrhea in *C. difficile* colitis. Toxin A induces epithelial cell necrosis and an inflammatory response in the lamina propria. Toxin B is typically “cytotoxic” to a wide variety of cells in tissue culture. Toxin B is a 250-kDa protein with considerable homology to toxin A and a common intracellular mechanism of action [2]. Both toxins induce the glucosylation of a threonine residue in rho proteins, which are small GTP-binding proteins that regulate cell shape through modulation of the actin cytoskeleton [3, 4]. The mechanism by which glucosylation of rho proteins induces fluid secretion is not known.

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

The Journal of Infectious Diseases 2001; 184:648–52
© 2001 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2001/18405-0019S02.00
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 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).

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 also was 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).

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 and results in destruction of the cells [10]; whether that process induces the release of factors that might induce Cox-2 expression in macrophages is not known. A more likely mechanism is that the induction of Cox-2 is a direct effect of toxin A on 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-
Figure 1. Cyclooxygenase (Cox)-2 immunohistochemistry of rabbit ileum. A, Normal ileum shows no Cox-2 expression. B, Ileum 2 h after Clostridium difficile toxin administration has stained lamina propria mononuclear cells in villi (arrow). C, Higher power view of a villus 2 h after C. difficile toxin A administration has stained lamina propria mononuclear cells that appear to be macrophages (small arrow) and linear structures in middle of villus that appear to be blood vessels (large arrow). D, Endothelial cell staining in submucosa of C. difficile toxin A–treated ileal tissue. Original magnifications: A and B, ×50; C and D, ×100.

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.

Acknowledgments

We thank Charlotte Martin, Leah Barrett, and Suzanne Schloemann for invaluable technical assistance and David Lyerly for providing Clostridium difficile toxin A for the rabbit ileal loop experiments.
References


