Reinforcement of the Intestinal Mucus Layer Protects Against *Clostridium difficile* Intestinal Injury In Vitro

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**BACKGROUND:** *Clostridium difficile* infection is increasing in incidence and severity. Attributable factors include virulence factors, including *C difficile* toxins A and B, as well as host immunologic status. The mucus component of the intestinal barrier is impaired by malnutrition, shock insults, and alterations in the gut microbiome. Exogenous phosphatidylcholine (PC) administration results in reinforcement of the mucus layer and is of therapeutic benefit in chronic ulcerative colitis. We therefore studied the role of exogenous PC combined with secretory immunoglobulin A (IgA) in intestinal barrier function against *C difficile* infection in vitro.

**STUDY DESIGN:** Dimeric IgA was placed in the basal chambers of mucus-producing (HT29-methotrexate) and non-mucus-producing (HT29) strains of intestinal epithelial monolayers and allowed to undergo transcytosis and, in additional experiments, exogenous colostral IgA (30 ng/mL) was added to the apical media. After subsequent coculture with PC and *C difficile* toxin A in the apical chamber, tumor necrosis factor-α, interleukin-6, toxin A uptake, intestinal epithelial cell monolayer permeability, and necrosis were determined.

**RESULTS:** A significant decrease of 4- to 5-fold in tumor necrosis factor-α and interleukin-6 levels and equally significant decreases in toxin A uptake and permeability changes in the intestinal cell monolayers with mucus or PC and transcytosed or colostral IgA vs control are shown. All groups analyzed also displayed a 2- to 3-fold reduction in necrosis.

**CONCLUSIONS:** Mucus or “exogenous” mucus in the form of PC has a synergistic role with secretory IgA in barrier defense against *C difficile* toxin A. Exogenous PC administration can be a therapeutic adjunct in patients with severe or recalcitrant *C difficile* infection. (J Am Coll Surg 2014;219:460–469. © 2014 by the American College of Surgeons)

*Clostridium difficile* infection (CDI) is the leading cause of health care-associated diarrhea. The clinical symptoms can range from asymptomatic carrier to self-limited diarrhea, to severe life-threatening colitis (toxic megacolon), and even death. Disease severity has been causally linked to *C difficile* virulence factors and the host immune and inflammatory responses. Two main virulence factors, toxin A and toxin B have been described for *C difficile.* Debate remains about the relative importance of these 2 toxins; however, it is likely that both toxin A and toxin B are important in CDI. Even with the emergence of certain hypervirulent *C difficile* strains (NAP1/027), it is now recognized that the host immune and inflammatory responses are the primary determinants of clinical outcomes in CDI.

Host defense against *C difficile* include both the innate and adaptive immune systems. Innate immunity of the mucosal surface at the gastrointestinal tract is provided by the intestinal epithelium and mucin-producing goblet cells, as well as antimicrobial peptides, lysozymes, and the commensal flora. Mucins act as the main structural component of the mucus layer, giving rise to its viscoelastic protective properties. Phospholipids account for a minor part of the mucus lipids. However the hydrophobic properties of phospholipids result in significant protective properties of intestinal mucus.

Secretory immunoglobulin A (SIgA) is the major antibody at mucosal surfaces. The primary function of SIgA is immune exclusion whereby access to the mucosal
surface by intestinal pathogens and luminal toxins including *C. difficile* toxins is limited. Secretory IgA is produced locally and reaches the luminal surface by way of a specialized receptor, the polyimmunoglobulin receptor. This process is dependent on the microtubule component of the intestinal epithelial cell (IEC) cytoskeleton. Of note, many of the adverse effects of *C. difficile* toxins are related to disruptions of the IEC cytoskeleton. This can result in loss of effective luminal SlgA levels.

In a previous study, we demonstrated that both the intestinal mucus layer and SlgA were important in limiting *C. difficile* toxin A-associated injury in an intestinal epithelial cell culture model. A synergistic effect of mucus and SlgA was also noted. Phosphatidylcholine (PC), a major component of the lipid compound of intestinal mucus, has been shown to have anti-inflammatory effects in clinical, animal, and in vitro studies. Decreased PC content in the intestinal mucus has been demonstrated in patients with ulcerative colitis. In this regard, symptomatic improvement in ulcerative colitis patients after administration of exogenous delayed-release PC has been reported. We postulate that passive immunization strategies at the gut level and the use of exogenous PC to “reinforce” the mucus layer can be adjunctive treatment in “at risk” patients with CDI. This was studied in our in vitro model.

**METHODS**

**Intestinal epithelial cells**

HT-29 cells were obtained from American Type Culture Collection and routinely cultured with Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 4.5 g/L glucose, and gentamicin in an atmosphere of 5% CO₂ at 37°C. Medium was changed twice a week and cells were passaged every 10 to 14 days. Cells (5 × 10⁵) were seeded on the apical surface of a polycarbonate membrane (3.0-μm pore size) (Transwell; Corning Costar Core) in a 2-chamber cell culture system and allowed to form polarized monolayers. When grown in this system, HT-29 cells form polarized monolayers that are confluent after 10 to 14 days in culture.

**HT29 methotrexate cells**

The HT29-methotrexate (MTX)-E12 cell line was obtained from HPA Cultures and routinely cultured with Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 4.5 g/L glucose, 1% nonessential amino acids (Gibco), and 1% antibiotic/antimycotic (Gibco) in an atmosphere of 5% CO₂ at 37°C. Medium was changed twice a week and cells were passaged every 10 to 14 days. Cells (5 × 10⁵) were seeded on the apical surface of a polycarbonate membrane (3.0-μm pore size) (Transwell; Corning Costar Core) in a 2-chamber cell culture system and allowed to form polarized monolayers. When grown in this system, HT29-MTX-E12 cells form polarized monolayers that are confluent after 10 to 14 days in culture.

**Experimental design**

Confluent non–mucus-producing (HT29) and mucous-producing (HT29-MTX) IEC monolayers were established in a 2-chamber cell culture system (CoStar). Dimeric IgA was placed in the basal chamber of the cell culture system and allowed to undergo transcytosis overnight, resulting in release of SlgA into the apical chamber. Total SlgA recovered from the apical chamber media was quantitated by ELISA. Exogenous PC (200 μM) was added to the apical chamber for 1 hour and the cells were subsequently challenged with *C. difficile* toxin A (50 μg/mL for 6 hours). Intestinal epithelial cell monolayer permeability and cell necrosis were then determined. The concentration of toxin A was based on our earlier study. Toxin A uptake was also quantified in both the nonmucus and mucus IECs. Expression of the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 was measured by collecting basal chamber supernatants post-toxin A exposure by ELISA. In a subset of experiments, colostral IgA (30 ng/mL), equivalent to the amount of transcytosed SlgA recovered, was added to the apical chamber(s) of IEC to compare the effect of exogenous administration of IgA with transported SlgA on the parameters of intestinal barrier function mentioned (Fig. 1).
hours. The cell monolayers were subsequently lysed with 10% Triton X-100 for 30 minutes at 37°C and the amount of intracellular toxin A was quantitated by ELISA. Briefly, ELISA plates were coated overnight with supernatants from the IEC lysates diluted in 0.05M sodium carbonate buffer (pH 9.6) at 4°C. The plate was washed 3 times with PBS and blocked with 0.05% PBS-Tween20 for 2 hours at room temperature. After blocking, the plates were coated with a 1:1000 dilution of a monoclonal anti-\textit{C difficile} toxin A antibody developed in mouse (Thermo Fisher Scientific) for 2 hours at room temperature. A rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase (Sigma) was subsequently added for 2 hours at room temperature and the plate was developed using 3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA purchased from Sigma. The color reaction was stopped with 1M H$_2$SO$_4$ and absorbance was measured at 450 nm using an ELISA plate reader. Purified \textit{C difficile} toxin A was used to create a standard curve for the determination of toxin A concentration contained in the supernatants collected from the IEC lysates.

**Measurement of necrosis of intestinal epithelial cells**

HT29 and HT29-MTX cells in the presence and absence of SlgA and/or exogenous PC were cocultured with toxin A and analyzed for necrosis. Briefly, cells were removed from the polycarbonate filter using PBS supplemented with 3 mM ethylenediamine tetraacetic acid. Cells were washed and incubated with propidium iodide for 15 minutes in the dark, then mixed with a 1× binding buffer and analyzed by flow cytometry within 1 hour for maximal signal. Results are expressed as percent necrosis compared with control cells. In a subset of experiments, exogenous IgA was added to the apical chamber of confluent IEC monolayers and its impact on necrosis vs transcytosed IgA was measured as outlined here.

**Permeability determination using fluorescein isothiocyanate dextran**

HT29 and HT29-MTX cells were exposed to exogenous PC for 1 hour and then \textit{C difficile} toxin A for up to 6 hours in the presence or absence of SlgA. Medium was subsequently removed and the monolayers washed. Fluorescein isothiocyanate (FITC)-dextran (10,000 molecular weight, 2.2 mg/mL in PBS) was added to the apical side of each insert, and the monolayers were incubated for 2 hours at 37°C. Monolayer permeability to FITC-dextran was then determined by measuring the fluorescence of the basal solution in each well at 485 nm (excitation wavelength) and 535 nm (emission wavelength). Permeability data were expressed as nmol cm$^{-2}$ h$^{-1}$. In a subset of experiments, exogenous IgA was added and its effect on the permeability of the FITC probe in the presence and absence of toxin A was assessed.
Cytokine analysis by enzyme-linked immunosorbent assay

Basal supernatants of HT29 and HT29-MTX cells in the presence and absence of transported IgA and treated with exogenous PC and/or toxin A for up to 6 hours were collected at the end of the experiments and immediately stored at −70°C for subsequent cytokine analysis. Interleukin-6 and TNF-α were quantitated in the previously frozen supernatant samples using a solid-phase sandwich ELISA. These immunoassay kits are commercially available and were used according to the manufacturer’s directions (Cytoscreen; Biosource International). The minimal detectable levels of IL-6 and TNF-α with these kits are 2 pg/mL for IL-6 and <1 pg/mL for TNF-α. In a subset of experiments, TNF-α and IL-6 levels were quantitated using exogenously administered IgA vs transcytosed IgA.

Statistical analysis

All samples were compared with an ANOVA with a post-hoc Tukey test. Statistical significance was inferred at p < 0.001. All data are expressed as mean ± SD.

RESULTS

HT29 intestinal epithelial subgroups are depicted in Figures 2 and 3. Tumor necrosis factor-α and IL-6 levels were decreased by 50% after apical delivery of SlgA after transcytosis overnight. The mucus layer present in the HT29-MTX strain decreased TNF-α and IL-6 an additional 50%. The substitution of PC for the mucus layer or the addition of exogenous SlgA to the apical surface has similar effects on cytokine levels vs the mucus-producing HT29 strain after IgA transcytosis. Tumor necrosis factor-α and IL-6 cytokine levels in HT29 cells in the absence of toxin A coculture were 6.7 ± 0.6 and 2.5 ± 0.7. HT29-MTX control levels of TNF-α and IL-6 were 12.1 ± 1.2 and 6.9 ± 0.9.

HT29 cell monolayer integrity was indexed by cellular necrosis, C difficile toxin A uptake and cell monolayer permeability. HT29 cellular necrosis decreased by 50% after transcytosis of SlgA. There were only slight improvements with either an intact mucus layer present in the HT29-MTX strain or by exogenous PC replacement of the mucus layer (Fig. 4). Control (no toxin A coculture) values of HT29 and HT29-MTX cellular necrosis were 6.2 ± 1.2 and 5.6 ± 1.0, respectively. The results obtained for C difficile toxin A uptake by HT29 cells are depicted in Figure 5. Transcytosed IgA alone decreased toxin A uptake by nearly 50%. Toxin A uptake was decreased approximately 50% more by either the presence of the mucus layer or PC replacement of the mucus layer. Immunoglobulin A administered passively into the apical chamber resulted in toxin A uptake similar to that which reached the HT29 apical cell surface by transcytosis. Permeability to FITC-dextran (4000 molecular weight) in the different HT29 treatment
groups is depicted in Figure 6. Secretory IgA alone had a minimal effect on HT29 cell permeability after *C. difficile* toxin A exposure. However, both mucus and PC, acting as a mucin substitute, acted synergistically with SIgA whether transcytosed or passively added to the apical surface of HT29 cells to significantly reduce HT29 cell permeability. Permeability in control cells was 0.34 ± 0.1 for HT29 and 0.45 ± 0.02 for HT29-MTX cells.

**Figure 3.** The synergistic effect of endogenous or exogenous mucus (phosphatidylcholine [PC]) and secretory immunoglobulin A (SIgA) on interleukin-6 (IL-6) expression in intestinal epithelial cells exposed to *Clostridium difficile* toxin A for 6 hours. Basal chamber supernatants were collected from HT29 or HT29-methotrexate (MTX) cells exposed to *C. difficile* toxin A (50 μg/mL) for 6 hours in the presence of SIgA and exogenous mucus (PC). Interleukin-6 was quantitated using an ELISA. Subsets of cells were exposed to exogenous IgA (30 ng/mL colostral IgA).

**Figure 4.** The effect of mucus and secretory immunoglobulin A (SIgA) on HT29 and HT29-methotrexate (MTX) cell necrosis in the presence of *Clostridium difficile* toxin A. Endogenous mucus or exogenous mucus in the form of phosphatidylcholine (PC) (200 μM for 1 hour) + SIgA had a synergistic effect on reducing intestinal epithelial cell (IEC) necrosis after exposure to *C. difficile* toxin A. Non—mucus-producing IECs treated with exogenous mucus (PC) and IgA resulted in a marked reduction in IEC necrosis comparable with the mucus-producing IECs exposed to transcytosed SIgA. MFI, mean fluorescence intensity.
DISCUSSION

*Clostridium difficile* infection is increasing in incidence, with a higher proportion of patients with fulminant colitis, toxic megacolon, and resultant death. In the past, this was attributed to the emergence of the virulent NAP1/027 strain, which produces 16- to 20-fold higher amounts of toxin A and toxin B. However, subsequent epidemiologic studies have shown that host responses, including markers of intestinal inflammation, and systemic humoral and proinflammatory responses, and not *C difficile* strain type or bacterial burden, correlate with clinical outcomes, including mortality.5,26,27

Figure 5. *Clostridium difficile* toxin A uptake in the presence of endogenous or exogenous mucus and immunoglobulin A (IgA). HT29 and HT29-methotrexate (MTX) cells were challenged by placing *C difficile* toxin A (50 ng/mL) in the apical chamber for up to 6 hours in the presence of mucus and secretory IgA. Cell monolayers were subsequently lysed and the amount of intracellular toxin A was quantitated by ELISA. PC, phosphatidylcholine.

Figure 6. The role of endogenous and exogenous mucus and immunoglobulin A (IgA) in HT29 or HT29-methotrexate (MTX) cell permeability in the presence of *Clostridium difficile* toxin A. The mucus-producing HT29-MTX cells and the presence of transcytosed secretory IgA resulted in protection of the intestinal barrier against *C difficile* toxin A exposure, as noted by the reduction in monolayer permeability to the fluorescein isothiocyanate-dextran (4000 molecular weight) (FD4) probe. Exogenous addition of PC (200 μM) and IgA (30 ng/mL colostral IgA) resulted in a similar reduction in FD4 permeability of the HT29 monolayer.
A number of clinical risk factors for *C. difficile* colonization and infection have been identified. These include previous antibiotic administration, acid suppressive therapy, cancer, inflammatory bowel disease, gastrointestinal surgery, recent hospitalization or admission to an extended care facility, and elderly patients. Common to these seemingly diverse patient populations is the likelihood of perturbations in either or both innate and acquired immune function at mucosal surfaces.

The first step in the initiation of CDI is binding of the toxin to colonic epithelial surface receptors. Internalization of toxin into the cytoplasm leads to a series of events, which leads to alterations in the actin cytoskeleton, disruption of epithelial barrier function, and apoptosis. Toxin A and B also both activate the inflammasome and nuclear factor-kB–mediated pathways, which results in proinflammatory cytokine and chemokine production. Subsequent signaling largely through IL-8 leads to a massive influx of PMNs and exacerbation of the injury to the intestinal barrier.

There is increasing recognition of the importance of host innate and humoral immune response to CDI. The cellular components of innate mucosal immunity include specialized secretory epithelial cells within the mucosa, ie, goblet cells and Paneth cells. Phosphatidylcholine, a major phospholipid in mucus, is primarily responsible for the protective hydrophobic surface of the colon. Other potential protective functions for luminal PC include intrinsic anti-inflammatory effects and the ability to modulate mucosal signaling via lipid rafts. Of note, PC is reduced in the mucus of patients with ulcerative colitis. Effective delivery of PC via enteric formulations has clinical efficacy in ulcerative colitis patients. This suggests that exogenous PC can reinforce the mucus layer and thereby prevent mucosal injury after venous noxious insults.

Mucins, larger glycoproteins secreted by goblet cells, are the primary organic component of the mucus layer. The mucus barrier not only provides a mechanical barrier to luminal pathogens and toxins, but also retains various antimicrobial peptides produced by Paneth cells, as well as SlgA and the secretory component of SlgA. Acquired immunity to *C. difficile* involves both systemic and mucus antibody responses. Johnson and colleagues reported a protective function of serum IgM and IgG against toxin A in the clinical outcomes after colonization or infection. Likewise, animal studies suggest that intravenous immunoglobulins can be protective against *C. difficile* toxin lethality. This is likely related to the association of systemic toxin A and B recovery with lethality in these animal models. *Clostridium difficile* infection begins at the colonic mucosal surface, therefore, the mucosal antibody response to *C. difficile* toxins is important in the front-line defense against CDI. Stubbe and colleagues have shown that polymeric IgA was superior to monomeric IgA or IgG (all toxin A specific) in preventing *C. difficile* toxin A damage to T84 cell monolayers. This likely reflects the greater binding ability of the polymeric form of the antibody. However, both SlgA (polymeric) and free secretory component can bind toxin A, and can also function as nonspecific “natural autoantibodies.”

In our previous in vitro cell culture model, we studied the ability of an intact mucus layer and/or SlgA to protect HT29 colonic epithelial cell monolayer from *C. difficile* toxin A injury. Both the mucus layer and IgA inhibited toxin A uptake and subsequent cellular injury and proinflammatory cytokine release after toxin A exposure. A synergistic effect was noted by the combination of the mucus layer and IgA against toxin A injury and inflammatory cytokine production. Toxin A was used in the current study as well. Notably, both toxin A and toxin B are important in the pathogenesis of *C. difficile* infection and can act synergistically. This was not addressed and is a limitation of this current study.

In the current study, we compared the barrier protective properties of intrinsic mucus vs PC as a mucus replacement. Similar barrier protection was afforded by PC vs the intrinsic mucus layer. We next compared the route of delivery of IgA with the mucosal surface of the HT29 cell monolayers. Intuitively SlgA arriving by way of transcytosis would likely be partly anchored in the mucus layer by way of binding to mucin components. The passively added SlgA would likely remain in the loose outer layer of the mucus layer. However, similar efficacy between transcytosed and passively added SlgA against toxin A when added at equivalent concentrations was noted. This might not be apparent in models using live *C. difficile* bacteria vs exposure to toxins, as performed in our model.

Effective innate and adaptive immune response to *C. difficile* inhibits toxin binding to intestinal epithelial cells, thereby limiting subsequent cytotoxic and enterotoxic effects. Exogenous PC and the “passive” addition of SlgA were equally efficacious as the intact mucus layer and transcytosed IgA in barrier function against toxin A. Effective delivery of PC and SlgA into the colon via the enteral route will require enteric coating to inhibit PC absorption in the small intestine and IgA cleavage from gastric acid and intestinal proteolysis. The structure of SlgA and its accompanying secretory component allow it to resist proteolytic cleavage in the harsh environment of the gut. Secretory IgA is the dominant immunoglobulin in colostrum and milk. Hyperimmune bovine...
colostrum can be produced by repeated immunization of pregnant dairy cows. This hyperimmune bovine colostrum has high titers of anti-toxin A and anti-toxin B antibodies. The use of hyperimmune bovine colostrum has shown efficacy in preliminary clinical studies.

CONCLUSIONS

Strategies to bolster the mucus layer of the colonic epithelial barrier and/or to enhance delivery of SIgA to the mucosal surface might prove to be effective adjuncts in the treatment of CDI. These strategies use the natural protective response against such infections, without the potential deleterious effects of antimicrobial therapy.

Author Contributions

Study conception and design: Diebel, Liberati
Acquisition of data: Liberati
Analysis and interpretation of data: Diebel, Liberati
Drafting of manuscript: Diebel, Liberati
Critical revision: Diebel, Liberati

REFERENCES

Discussion

INVITED DISCUSSANT: DR EUGENE CHOI (Chicago, IL): I would like to thank the Western Surgical Association for the opportunity to discuss this article.

The authors present data to help our understanding and possible treatment strategy for a rampant cause of opportunistic infection in not only surgical patients, but also all hospitalized patients. *Clostridium difficile* is a species of gram-positive spore-forming bacterium that is responsible for causing antibiotic-associated diarrhea. Disruption of the normal bacterial flora and overpopulation of *C. difficile* is responsible for the release of toxins, which can cause bloating and diarrhea, pseudomembranous colitis, and, in extreme cases, life-threatening toxic megacolon. In addition, hospital-onset *C. difficile*–associated diarrhea has been associated with longer length of stay and higher hospital costs.

This article presents an extension of previously published work into mature goblet cells using MTX. The HT29 intestinal epithelial subgroups did not include evaluation of HT29-MTX alone or HT29-MTX with PC or HT29 with PC. Is there a difference in permeability, necrosis, and expression of TNF and IL-6 with toxin A expression between HT29 and HT29-MTX-E12 without IgA or PC treatment? Are the effects on permeability, necrosis, and expression of TNF and IL-6 greater when the HT29-MTX-E12 are supplemented with PC and IgA? In addition, does HT29 supplemented with PC alone have effects on permeability, necrosis, and expression of TNF and IL-6 similar to HT29 with IgA alone?

2. Mucus is a protection barrier, are there other components in the mucus that also have a protection affect on *C. difficile* and have you evaluated them?

3. Phosphatidylcholine can constitute mucus and act as a barrier against toxin A; however, PC also plays a role in membrane-mediated cell signaling. Has this been evaluated? If the goal of PC and IgA is to prevent inflammation associated with toxin A, is there a role for probiotics, which are thought to decrease inflammation and modulate the immune system?

DR LAWRENCE N DIEBEL

Regarding the different subtypes of the HT29 cells, in preliminary work, we demonstrated no difference in the inflammatory potential of HT29 cells, whether they are mucus producing or non—mucus producing.

Regarding other members of the mucus layer that are important, well, one thing I did not discuss earlier is that *C. difficile* toxin does decrease the exocytosis of the mucin component of the mucus layer, and a similar effect has been noted with Tagamet. So there might be an effect outside of PC. But the bottom line is that the mucus layer does not work effectively in *C. difficile* patients.

Yes, about PC, does it have any other effects besides at the barrier, and what we need to do now with that is to look at the function of inflammasomes and nuclear factor kB signaling inside the cell to answer that, because PC probably works at 3 different levels, at the gut surface level, by acting as a mucus layer, but also there is cellular uptake. So it can affect the cell intracellularly, and by way of lipographs, and affect cell signaling. So we have to look at the inflammasome and nuclear factor kB signaling to answer that question. Probiotics is an interesting question. I think probiotics alone in a leaky gut is a bad situation; and there are case reports of severe fungal infections developing when you are talking about *Saccharomyces*, which actually does affect *C. difficile*. So I think if you are going to use probiotics, I think there are some limited data; I am aware that when using probiotics with an immunoglobulin component it can be helpful to add a PC or some mucus substitute to that, but probiotics alone in a sick patient with *C. difficile* is a bad idea.

And regarding PC, I have a patient that has had recurrent *C. difficile* colitis. He is a Michigan State police officer who was injured and is now quadriplegic. I put a diaphragm pacer in, so he is no longer on a ventilator, but he has had recurrent bouts of *C. difficile*. I told him I was doing research about this, and I told him PC is the answer. But the commercially available forms, if you take it by pill or any other formula, are absorbed in the intestine, so none of it reaches the colon. So you need an extended-release formula, which...