Differential Effects of Varying Concentrations of Clostridium difficile Toxin A on Epithelial Barrier Function and Expression of Cytokines

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Background. Presentation after Clostridium difficile infection may depend on the level of epithelial exposure to toxins. We investigated epithelial barrier function and expression of interleukin (IL)–8 and transforming growth factor (TGF)–β in response to varying concentrations of C. difficile toxin A.

Methods. T84 cells were either preexposed or continuously exposed to C. difficile toxin A (0.01–1000 ng/mL). Barrier function was assessed by measurements of transepithelial electrical resistance.

Results. Preexposure to ≤10 ng/mL toxin A led to an increase in the release of TGF-β1, but there was no change in the expression of IL-8. In contrast, after preexposure to >10 ng/mL toxin A, there was enhanced expression of IL-8, but release of TGF-β1 was similar to that in control monolayers. After preexposure to >10 ng/mL toxin A, there was complete and irreversible loss of electrical resistance. At lower concentrations, loss of resistance across monolayers was followed by recovery, which was enhanced by all 3 recombinant isoforms of TGF-β. Pretreatment with recombinant isoforms of TGF-β or coculture with TGF-β3–expressing colonic subepithelial myofibroblasts was also protective.

Conclusions. In C. difficile infection, the development and severity of colonic inflammation may depend on the exposure of intestinal epithelial cells to toxins and the expression of proinflammatory (IL-8) and protective (TGF-β) factors.

Clostridium difficile is a spore-forming gram-positive anaerobic bacillus and is the most commonly encountered bacterial enteropathogen in hospitalized patients. It is recognized that some patients colonized with toxigenic C. difficile remain asymptomatic [1]. In those who develop disease, clinical presentation can range from mild diarrhea to life-threatening pseudomembranous colitis [2, 3]. Histological examination of colonic mucosal samples from patients with C. difficile–associated colitis frequently shows that the pseudomembrane characteristically arises from a small area of superficial ulceration, in association with infiltration by polymorphonuclear cells [4]. Often, in the mucosa adjacent to the area of ulceration, the enterocytes and the underlying lamina propria appear to be normal.

The reasons for the different types of clinical presentation and focal mucosal inflammation (in patients with colitis) after colonization with toxigenic C. difficile remain unknown. It is likely that they represent the outcome of cellular and molecular interactions between the host mucosal defense mechanisms and C. difficile toxins A and B. The first host mucosal cells that the toxins would interact with in the colon are epithelial cells, which provide an important barrier to luminal products via intercellular junctional complexes. Paracellular permeability is regulated by tight junctions, which are located apically between epithelial cells [5]. A number of inflammatory diseases of the intestine are characterized by disruption of the function of tight junctions, leading to increased permeability to luminal contents. Cytokines, such as transforming growth factor (TGF)–β and others derived from enterocytes and from cells of the underlying lamina propria, have been shown...
to be capable of regulating paracellular permeability of epithelial monolayers via their effects on tight junctions [6]. After interactions with pathogenic microorganisms or their products, epithelial cells are induced to express a number of cytokines, of which interleukin (IL)–8, the potent chemoattractant of polymorphonuclear cells, has been studied the most [7]. Expression of IL-8 is likely to be an important early response by epithelial cells, initiating the cascade of events that leads to an acute inflammatory response.

*Clostridium difficile* toxins A and B have been shown to induce disruption of epithelial barrier function [8], and toxin A has also been shown to induce the expression of IL-8 [9–12]. The ultimate fate of the epithelial cells is death by apoptosis. Intracellularly, the toxins have been shown to monoglucosylate the Rho family of GTP-binding proteins [13], with the subsequent enhancement of epithelial paracellular permeability via disruption of tight junctions and the actin cytoskeleton [8].

In most of the studies reported to date, only high (>10 ng/mL) concentrations of toxin A have been used, and responses to low concentrations of the toxin remain to be fully characterized. Moreover, the ability of epithelial barrier function to recover after brief exposure to toxin A (which may occur in vivo) and factors that may regulate such recovery are unknown. In the present study, we show that, after 3 h of preexposure to ≤10 ng/mL purified toxin A, there was an increase in the release of TGF-β1 by T84 cells, but there was no change in the expression of IL-8. In contrast, 3 h of preexposure to >10 ng/mL toxin A led to enhanced expression of IL-8, but the release of TGF-β1 was similar to that in control monolayers. Within 24 h after preexposure (for 3 h) to >10 ng/mL toxin A, there was complete and irreversible loss of electrical resistance across T84 monolayers. At lower concentrations, loss of resistance across monolayers was followed by recovery that was dependent on the concentration of toxin originally applied. Pretreatment with recombinant isoforms of TGF-β or coculture with human colonic myofibroblasts led to significantly lower decreases in electrical resistance in response to toxin A. In addition, TGF-β enhanced recovery in electrical resistance in response to toxin A. These studies suggest that the level of colonic epithelial exposure to *C. difficile* toxins may determine the mucosal and, therefore, clinical response.

**MATERIALS AND METHODS**

**Purification of toxin A.** Toxin A was purified as described elsewhere [14]. In brief, toxigenic *C. difficile* (strain VPI 10643) was cultured in dialyzing tubing, and culture filtrates were subsequently applied to thyroglobulin affinity column and 2 sequential anion exchange column chromatography steps, by use of Q-Sepharose FF and Mono Q columns (Amersham Biosciences).

**T84 cell culture.** T84 colonic epithelial cell line was obtained from the European Collection of Animal Cell Cultures (ECACC), and studies were performed at passage 60–69. Cells were cultured at 37°C in 5% CO₂ and maintained in 50% Dulbecco’s minimal essential medium (DMEM)/50% Ham’s F-12 medium (Gibco BRL), supplemented with 10% fetal calf serum (FCS; Gibco BRL), 2 mmol/L glutamine (Sigma), 100 U/mL penicillin (Britannia Pharmaceuticals), and 0.1 mg/mL streptomycin (Evans Medical).

For studies of release of TGF-β and IL-8, T84 cells were grown to confluence in 24-well tissue plates (Nunc; Gibco BRL). On reaching confluence, cells were incubated in 0.1% FCS/DMEM for 24 h. *C. difficile* toxin A was added, to give final concentrations of 0.01, 0.1, 1, 10, 100, or 1000 ng/mL, to each well for 3 h. The untreated and toxin-exposed cells were washed with prewarmed (to 37°C) 0.1% FCS/DMEM on 8 occasions, to remove any remaining extracellular toxin. Toxin A can interfere with the TGF-β bioassay and, therefore, its removal from the extracellular compartment, after preexposure of T84 cells for 3 h, allowed accurate determination of the release of TGF-β in the cell supernatant samples during subsequent culture of the epithelial cells. Lack of toxin A in the final wash (after 3 h of preexposure) was confirmed by use of the highly sensitive Vero cell assay [14]. After 3 h of preexposure to toxin A, T84 cells were cultured for a further 48 h. Supernatant samples were obtained during 2 periods, 0–24 h and 24–48 h. After centrifugation (at 13,000 g for 10 min) and microfiltration (using a 0.2-μm filter), these samples were stored at −70°C until assayed for TGF-β and IL-8.

**TGF-β bioassay.** To determine the concentration of TGF-β in supernatant samples obtained from T84 cells preexposed to toxin A, a specific bioassay was used [15, 16]. This bioassay is based on the ability of TGF-β to inhibit proliferation of the mink lung epithelial cell line Mv1Lu (obtained from ECACC). Mv1Lu cells (in 0.1% FCS/DMEM) were seeded at 5 × 10⁴ cells/well in 24-well Nunc plates. After 3 h, recombinant human TGF-β1 (R&D Systems) or supernatant samples of T84 cells preexposed to control and toxin A were added. To determine the content of total TGF-β (bioactive plus latent forms), 1 aliquot of supernatant samples was treated with concentrated HCl, to a pH of 2, for 1 h [17], followed by neutralization with NaOH and HEPES.

Mv1Lu cells were incubated (at 37°C in 5% CO₂) for a total of 25 h, the final 4 h in the presence of [³H]methyl-thymidine (Amersham). Cells were fixed in methanol:acetic acid (3:1) for 1 h at room temperature. After 2 washes in 80% methanol, the cells were lysed with 1 mol/L NaOH for 1 h. Assays were performed in triplicate, and uptake of [³H]-thymidine was deter-
minded by use of a liquid scintillation counter (LKB β-counter; Wallac).

To quantify the isoforms of TGF-β present, acid-treated samples of T84 cells preexposed to control and toxin were incubated with 3.33 μg/mL neutralizing antibodies to either TGF-β1, TGF-β2, or TGF-β3 (R&D Systems) for 2 h before supernatant samples were added to Mv1Lu cells. We have previously demonstrated specificities of these isoform-specific neutralizing antibodies [18].

**IL-8 ELISA.** The supernatant samples harvested from confluent T84 monolayers preexposed to toxin A were examined for the presence of IL-8 by use of a specific sandwich ELISA (R&D Systems). In our laboratory, the inter- and intra-assay variation for this assay was <12%.

**Assay for mitochondrial dehydrogenase activity.** This assay was used to assess cell viability. Metabolism by mitochondrial dehydrogenase of the yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt [MTS(a)] gives a purple/brown formazan product that can be quantified spectrophotometrically.

T84 cells were grown to confluence in 96-well Nunc plates, before 3 h of exposure to toxin A, followed by washes in 0.1% FCS/DMEM, as described above. MTS assay (Sigma) was subsequently performed in triplicate, every 24 h for 6 days, by adding 20 μL of neat MTS reagent directly to sequential culture wells containing 100 μL of 0.1% FCS/DMEM. After incubation with MTS for 4 h, spectrophotometric absorbance of the samples was performed by use of a micrometer plate reader.

**Electrophysiological studies of T84 cells.** Experiments were performed to examine the effect of toxin A on electrical resistance across T84 monolayers. Transwells with 12-cluster plates of 12 mm insert diameter (Costar), giving a growth area of 1 cm², were used. Epithelial cells were seeded at 5 × 10⁴ cells/well in the upper compartment and incubated at 37°C in 5% CO₂. Medium was replaced in both compartments twice a week with 10% FCS/DMEM (Gibco BRL). The volumes of media in the apical and basal compartments were 0.5 mL and 1.5 mL, respectively. T84 cell monolayers were grown until their resistance measurements were >1000 Ω/cm², by use of an Epithelial Volt-Ohmmeter (World Precision Instruments), when they were considered to be “electrically confluent.” Cell monolayers were then incubated with 0.1% FCS/DMEM (Gibco BRL) for 24 h. Toxin A was applied to the apical compartment, to give final concentrations of 0.01, 0.1, 1, 10, 100, or 1000 ng/mL. For studies of preexposure to toxin, any remaining extracellular toxin was removed after 3 h, as described above. In some experiments, recombinant isoforms of TGF-β were added to the apical compartment of T84 monolayers, either 24 h before continuous exposure to toxin A or, in experiments of preexposure to toxin, after the final wash. All measurements of electrical resistance were performed 3 times in duplicate.

**RESULTS**

**Release of TGF-β from monolayers of T84 cells preexposed to toxin A.** Acid-treated supernatant samples obtained during the first 24 h showed a significant increase in the release of bioactive TGF-β from T84 cells preexposed to 0.01 (P<.05), 0.1 (P<.01), 1 (P<.001), or 10 (P<.001) ng/mL toxin A, compared with controls (figure 1A). However, after preexposure to ≥100 ng/mL toxin A, release of TGF-β from T84 cells was similar to that from controls. Studies using non–acid treated samples showed that most of the TGF-β released was in the latent form. For supernatant samples obtained during the 24–48-h period (figure 1B), the pattern of release of total TGF-β was similar to that during the first 24 h.

Studies using specific neutralizing antibodies to isoforms of TGF-β showed that TGF-β1 was the predominant isoform expressed by T84 cells after treatment with toxin A (figure 2), with a significant increase in TGF-β1 at 0.1 (P<.01), 1 (P<.001), and 10 (P<.05) ng/mL toxin A (but not at higher concentrations of toxin). There was no significant change in the production of either TGF-β2 or TGF-β3.

**The effect of pretreatment with toxin A on release of IL-8.** Release of IL-8 from T84 cells preexposed for 3 h to different...
Figure 1. Induction of expression of transforming growth factor (TGF)–β by T84 cells, in response to *Clostridium difficile* toxin A. T84 monolayers were preexposed (for 3 h) to varying concentrations of toxin A, followed by extensive washing before culture in 0.1% fetal calf serum/Dulbecco’s minimal essential medium. Supernatant samples were subsequently obtained during the 0–24-h (A) and 24–48-h (B) periods. Concentrations of TGF-β were determined by bioassay using acid-treated (followed by neutralization, for total TGF-β bioactivity) and non–acid treated supernatant samples. Results are presented as mean (± SEM) of 4 experiments performed in triplicate. *P<.05, †P<.01, and *P<.001, vs. control.

concentrations of toxin A was examined by use of the same supernatant samples that were used for the TGF-β bioassay. During the first 24 h, there was no increase in the release of IL-8 from cells that had been preexposed to ≤10 ng/mL toxin A, but there was a significant increase in the release of the cytokine after preexposure to 100 ng/mL (*P<.004; figure 3). For supernatant samples obtained during the 24–48-h period, there was no increase in production of IL-8 at any of the concentrations of toxin A studied (data not shown).

**MTS assays in T84 cells preexposed to toxin A.** To determine the effect of 3 h of preexposure to toxin A on the metabolic activity of T84 cells, mitochondrial dehydrogenase activity was assessed by use of an MTS assay. At the concentrations studied, toxin A did not cause a change in metabolic activity during the first 24-h period (figure 4). During the 24–48-h period, there was a sharp decrease in the mitochondrial enzyme activity of cells preexposed to 1000 ng/mL (*P<.001) toxin A. T84 cells preexposed to <1000 ng/mL toxin A did not show a change in mitochondrial dehydrogenase activity, despite culture for 144 h.

**Changes in electrical resistance across T84 monolayers continuously exposed to toxin A.** The effect of continuous exposure to different concentrations of toxin A on electrical resistance across T84 cells was examined (figure 5). Concentrations ≥10 ng/mL led to rapid and significant decreases in resistance, with all showing statistically lower measurements than those for the control, at 3 h (*P<.05). For lower concentrations of toxin A, the decrease in resistance reached statistical significance at 24 h, for 1 and 0.1 ng/mL toxin A, and at 48 h, for 0.01 ng/mL toxin A (all *P<.04).
Figure 3. Expression of interleukin (IL)–8 by T84 cells, in response to Clostridium difficile toxin A. T84 monolayers were preexposed to toxin A (for 3 h). Supernatant samples were subsequently obtained during the 0–24-h period. Concentrations of IL-8 were determined by ELISA. Results are presented as mean (±SEM) of 4 experiments performed in duplicate.

*P < .004, vs. control.

Figure 4. Mitochondrial dehydrogenase activity in T84 cells exposed to Clostridium difficile toxin A. The 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]2H-terazolium assay was performed (as described in Materials and Methods) in duplicate on 3 occasions, on T84 monolayers preexposed to toxin A (for 3 h). *P < .001, vs. control. OD_{492}, optical density at 492 nm.

Effect of rTGF-β isoforms on electrical resistance across T84 monolayers continuously exposed to toxin A. T84 monolayers were incubated with 1 or 5 ng/mL rTGF-β1 for 24 h before the addition of toxin. Resistance measurements were then compared with those for toxin-exposed monolayers that had not been treated with rTGF-β1, in the same experiment. At 1 ng/mL rTGF-β1, there was a trend toward increased resistance values, compared with controls, for ≤10 ng/mL toxin A, but these did not reach statistical significance (data not shown). Compared with that across controls, 24 h of preincubation with 5 ng/mL rTGF-β1, -β2, and -β3 led to significantly greater electrical resistance across monolayers continuously exposed to ≤10 ng/mL toxin A (figure 6A–6C). For cells exposed to >10 ng/mL toxin A, there was no difference in resistance across any of the monolayers treated with TGF-β isoforms (compared with that across medium-only controls), and these data are not presented in the figures.

Changes in electrical resistance across T84 monolayers after preexposure to toxin A. Preexposure (for 3 h) of T84 monolayers to toxin A led to a decrease in electrical resistance that was dependent on the concentration of toxin originally applied (figure 7). Preexposure to ≥10 ng/mL toxin A lead to rapid (P < .05, vs. control at 3 h) and irreversible loss of electrical resistance. After preexposure to lower (<10 ng/mL) concentrations of toxin A, the decrease in resistance was of a much lower magnitude and occurred during a longer period. Moreover, this electrical resistance subsequently recovered over time. For cells preexposed to 0.01 ng/mL toxin A, this recovery was complete at 96 h.

Effect of isoforms of rTGF-β on electrical resistance across T84 monolayers preexposed to toxin A. The ability of TGF-β to influence the recovery of epithelial electrical resistance was studied by applying 1 ng/mL rTGF-β1, -β2, and -β3 to T84 monolayers preexposed to toxin A. After preexposure to ≤10 ng/mL toxin A, electrical resistance was significantly greater across T84 monolayers incubated with recombinant isoforms of TGF-β than across control monolayers (figure 8A–8C). The TGF-β–induced increase in resistance was especially prominent at later time points, when recovery was occurring in monolayers preexposed to lower concentrations of toxin A (figure 7). None of the recombinant isoforms of TGF-β had any effect on electrical resistance across T84 monolayers preexposed to ≥100 ng/mL toxin A.

Role of colonic myofibroblasts on toxin A–induced changes in transepithelial resistance. Human colonic subepithelial myofibroblasts have been shown to release bioactive TGF-β [22]. The effect of myofibroblasts on electrical resistance across T84 monolayers exposed to toxin was examined. After exposure to ≤10 ng/mL toxin A, electrical resistance across cocultures of T84 cells and myofibroblasts was significantly greater than
C. difficile

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Figure 5. Changes in electrical resistance across T84 monolayers continuously exposed to Clostridium difficile toxin A. Varying concentrations of toxin A were added to "electrically confluent" T84 monolayers grown on permeable inserts. Electrical resistance across the monolayers was measured at the time point indicated on the horizontal axis. Results are presented as mean (±SEM) of 3 experiments performed in duplicate.

*P<.05 and *P<.01, vs. control.

Figure 6. Effect of recombinant (r) isoforms of transforming growth factor (TGF)-β on resistance across T84 monolayers continuously exposed to Clostridium difficile toxin A. Confluent monolayers of T84 cells were cultured for 24 h in the presence of 5 ng/mL rTGF-β1 (A), rTGF-β2 (B), or rTGF-β3 (C), before continuous exposure to varying concentrations of toxin A. Electrical resistance across T84 monolayers was measured at specific time points and found to be increased or unchanged, compared with that across medium-only (without TGF-β) controls. The data are therefore presented in the figure as percentage increase in resistance across TGF-β isoform–treated monolayers, compared with that across medium-only controls, at the time point indicated on the horizontal axis. The data are expressed as mean (±SEM) of 3 experiments performed in duplicate. *P<.05, vs. medium-only control.

DISCUSSION

We and others have previously shown that C. difficile toxin A induces the release of IL-8 by T84 and HT29 human intestinal epithelial cell lines [9, 11]. In the present study, we have shown that 3 h of preexposure to high (≥100 ng/mL), but not low, concentrations of toxin A also induced release of IL-8 by T84 cells. In addition to induction of release of IL-8, preexposure to high concentrations of toxin A also led to a rapid decrease that across T84 monolayers (figure 9). This difference was especially prominent at earlier time points (3–48 h). When T84 cells were exposed to ≥100 ng/mL toxin A, myofibroblasts had no effect on electrical resistance.

In the T84–myofibroblast cocultures, the myofibroblasts, by themselves, did not contribute to electrical resistance, as illustrated by the fact that resistance across monolayers of myofibroblasts was 115 (±4) Ω/cm², compared with 114 (±5) Ω/cm² across membrane filter alone.

Colonic myofibroblasts enhance epithelial electrical resistance via TGF-β3. Conditioned medium from human colonic myofibroblasts enhanced transepithelial resistance across T84 cells (figure 10). The MFCM-induced increase in transepithelial resistance occurred via TGF-β3, as shown by the use of isoform-specific neutralizing antibodies.

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Figure 7. Changes in electrical resistance across T84 monolayers, in response to preexposure to Clostridium difficile toxin A. Confluent T84 monolayers (grown on permeable inserts) were preexposed (for 3 h) to varying concentrations of toxin A and, after extensive washing, were cultured in medium. Serial measurements of electrical resistance were made at the time points indicated on the horizontal axis. Results are presented as mean (± SEM) of 3 experiments performed in duplicate. Preexposure of T84 cells to ≥10 ng/mL toxin A resulted in rapid and irreversible decrease in resistance. Preexposure to <10 ng/mL toxin A led to mild-moderate decrease in resistance, followed by recovery that was inversely related to the concentration of toxin initially applied. * P < .05, † P < .01, and ‡ P < .001, vs. control.

in electrical resistance across T84 monolayers. During subsequent prolonged culture, there was no evidence of recovery in electrical resistance, which, for cells preexposed to 1000 ng/mL toxin A, is due to loss of cell viability (as illustrated by the loss of mitochondrial dehydrogenase activity). It is of interest that mitochondrial dehydrogenase activity in T84 cells preexposed to <100 ng/mL toxin A did not decrease during subsequent culture. This profile of mitochondrial enzyme activity is similar to that during continuous exposure of T84 cells to the toxin, observed by us elsewhere [9]. It is conceivable that transient (for a few hours) rather than continuous exposure of colonic epithelial cells to C. difficile toxins occurs in vivo. Our studies of epithelial responses after preexposure to toxin A have enabled us to not only assess the subsequent release of TGF-β by use of a bioassay (presence of the toxin would have interfered with this assay) but also to demonstrate the ability of epithelial monolayers to recover barrier function if exposure to low concentrations of the toxin is not maintained. Thus, in T84 monolayers preexposed to lower concentrations (<10 ng/mL) of toxin A, the decrease in transepithelial resistance was gradual (compared with higher concentrations) and was followed by recovery during subsequent culture (in the absence of toxin). The latter recovery in transepithelial resistance was significantly enhanced during subse-

Figure 8. Effect of recombinant (r) isoforms of transforming growth factor (TGF)-β on recovery in electrical resistance across T84 monolayers preexposed to Clostridium difficile toxin A. Confluent T84 monolayers (grown on permeable inserts) were preexposed (for 3 h) to varying concentrations of toxin A and, after extensive washing, were cultured in medium only or in the presence of 1 ng/mL rTGF-β1 (A), rTGF-β2 (B), or rTGF-β3 (C). The figure shows percentage increase in electrical resistance across monolayers treated with TGF-β isoforms (compared with that across medium-only controls), at the time points indicated on horizontal axis. For cells preexposed to >10 ng/mL toxin A, there was no difference in resistance across any of the monolayers treated with TGF-β isoforms (compared with that across medium-only controls), and these data are not presented in the figure. The data are expressed as mean (± SEM) of 3 experiments performed in duplicate. * P < .05, vs. medium-only control.
by all 3 recombinant isoforms of TGF-β. Of interest, preexposure to low concentrations of toxin A also induced expression of TGF-β by T84 cells, which peaked after exposure to 1 ng/mL toxin A and persisted over the course of 48 h. In contrast to IL-8, expression of TGF-β was not induced after exposure to high concentrations of toxin A. Of the 3 isoforms of TGF-β, T84 cells expressed mainly TGF-β1, which was present predominantly in the biologically inactive, latent form. We postulate that, in vivo, activation of the latent form of TGF-β would be mediated by the underlying lamina propria cells, such as myofibroblasts and macrophages. In addition to facilitating the recovery of epithelial barrier function of cells exposed to toxin A, mature TGF-β would also be expected to enhance epithelial restitution [22–24], which describes a process whereby cells adjacent to an area of ulceration migrate to reestablish epithelial continuity and barrier function. Indeed, restituting epithelial cells are often seen adjacent to an area of ulceration in biopsies of patients with pseudomembranous colitis (authors’ unpublished observations).

Our studies have also shown that pretreatment with TGF-β makes epithelial monolayers more resistant to the barrier-disrupting effect of continuous exposure to low concentrations of toxin A. Such an effect was also mediated by human colonic myofibroblasts, during coculture with T84 monolayers, and is likely to be due to bioactive TGF-β3 secreted by these cells. It should be noted that neither recombinant TGF-β nor colonic myofibroblasts had any effect on the rapid and irreversible loss of transepithelial resistance that occurred in the presence of high concentrations of toxin A. Previous studies have shown that the toxin A–mediated loss of transepithelial resistance occurs because of an impairment of the function of intercellular tight junctions [8, 25]. Both C. difficile toxin A and B have a similar intracellular mode of action, that of monoglucosylation and thereby inactivation of the Rho family of GTPases [26].
which are important in the regulation of the function of intestinal epithelial tight junctions [27]. Our studies suggest that exposure to low concentrations of toxin A leads to reversible impairment of the function of tight junctions, which can be modulated by TGF-β.

Pretreatment with TGF-β1 has also been shown to ameliorate the barrier-disrupting effects of Cryptosporidium parvum [28] and T cell–derived cytokines, such as interferon-γ, IL-4, and IL-10 [29]. Myofibroblasts, which in vivo are normally present immediately subjacent to the epithelial monolayer [19, 30], are an important source of bioactive TGF-β in the normal and chronically inflamed intestine [18], and our studies and those of Roche et al. [28] suggest a major role for these cells, not only in maintaining the integrity of the intestinal epithelium in vivo but also in providing protection against and facilitating the reestablishment of epithelial barrier function after injury induced by pathogenic microorganisms.

Previous studies have shown that intestinal trefoil factor (ITF), which is normally expressed by goblet cells in the small and large bowel [31, 32], and mucin glycoproteins protect against the barrier-disrupting effect of C. difficile toxin A [33]. In the latter study, combined application of mucin glycoproteins and ITF led to almost-complete prevention of toxin A–mediated loss of barrier function. The protective effect was seen only if the mucin glycoproteins and/or ITF were applied at the same time as the toxin, and not if they were added at a later time point.

The differential effects of varying concentrations of C. difficile toxin A in the induction of IL-8 (a proinflammatory cytokine, induced by high concentrations of the toxin) and TGF-β (a “protective” cytokine, induced by low concentrations of the toxin) are of interest. Our previous studies have shown that Bacteroides fragilis enterotoxin, which also impairs epithelial barrier function [34, 35] via an extracellular proteolytic action [36], induces the expression of both IL-8 and TGF-β1 in T84 cells, in a dose-dependent manner [37]. This suggests that the differential response to C. difficile toxin A seen in the present study is likely due to the specific intracellular effects of the toxin.

The results of our studies lead us to suggest a possible mechanism that could explain the histological changes seen in pseudomembranous colitis: focal areas of epithelial ulceration associated with “eruptions” of inflammatory cells and exudates. Local high concentrations of C. difficile toxins, which may occur because of the presence of a number of toxigenic C. difficile bacteria present close to a small area of the epithelium, would lead to expression of IL-8 and a focal loss of epithelial cells, due to detachment from the basement membrane and apoptosis [9, 38, 39]. Access of toxins to the lamina propria macrophages would induce the expression of tumor necrosis factor–α [40], which, together with IL-8, would initiate an inflammatory response. Epithelial cells in the area adjacent to the ulceration would be exposed to lower concentrations of the toxins, inducing the expression of TGF-β, which would induce epithelial restitution and recovery of barrier function. Epithelial and myofibroblast–derived TGF-β would also enhance resistance to barrier-disrupting effects of the toxins in those areas of the epithelium that have not yet been exposed to them. It is conceivable that the combined host-protective effects of ITF, mucin glycoproteins, and TGF-β from the epithelial cells and underlying myofibroblasts provide adequate protection, such that the “trigger point” of an inflammatory response is not reached, leaving these individuals asymptomatic. Thus, the level of secretion of toxin by the bacterium and host-protective factors may determine the mucosal (and, therefore, clinical) response in individuals infected with toxigenic C. difficile.

References


