MAJOR ARTICLE

Intestinal Epithelial Restitution After TcdB Challenge and Recovery From *Clostridium difficile* Infection in Mice With Alanyl-Glutamine Treatment

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Background. *Clostridium difficile* is an anaerobic bacterium that causes antibiotic-associated diarrhea. It produces toxin A and toxin B (TcdB), which cause injury to the gut epithelium. Glutamine is a fundamental fuel for enterocytes, maintaining intestinal mucosal health. Alanyl-glutamine (AQ) is a highly soluble dipeptide derivative of glutamine. We studied whether administration of AQ ameliorates the effects of TcdB in the intestinal cells and improves the outcome of *C. difficile* infection in mice.

Methods. WST-1 proliferation and cell-wounding-migration assays were assessed in IEC-6 cells exposed to TcdB, with or without AQ. Apoptosis and necrosis were assessed using Annexin V and flow cytometry. C57BL/6 mice were infected with VPI 10463 and treated with either vancomycin, AQ, or vancomycin with AQ. Intestinal tissues were collected for histopathologic analysis, apoptosis staining, and determination of myeloperoxidase activity.

Results. AQ increased proliferation in intestinal cells exposed to TcdB, improved migration at 24 and 48 hours, and reduced apoptosis in intestinal cells challenged with TcdB. Infected mice treated with vancomycin and AQ had better survival and histopathologic findings than mice treated with vancomycin alone.

Conclusions. AQ may reduce intestinal mucosal injury in *C. difficile*–infected mice by partially reversing the effects of TcdB on enterocyte proliferation, migration, and apoptosis, thereby improving survival from *C. difficile* infection.

Keywords. *C. difficile*; alanyl-glutamine; diarrhea.

Clostridium difficile is a gram-positive anaerobic bacterium that causes antibiotic-associated diarrhea and colitis. It is the leading cause of nosocomial diarrhea in North America. *C. difficile* infection results in longer length of hospital stay, increased costs, and excess morbidity and mortality in hospitalized patients [1]. The disease ranges in severity from asymptomatic carriage to severe clinical disease, such as profuse diarrhea, pseudomembranous colitis, toxic megacolon, and even death [2]. Infection with *C. difficile* is no longer confined to hospitalized individuals. The bacterium is now recognized as a cause of community-acquired diarrhea, with many but not all patients showing typical risk factors, such as use of antibiotics [3].

C. difficile acts mainly through its products, toxin A (TcdA) and toxin B (TcdB). However, in up to 36% of *C. difficile* isolates, a third toxin, called *C. difficile* transferase or binary toxin (CDT), is expressed, frequently in hypervirulent strains (such as Nap1/027) [4]. TcdA and TcdB inactivate Rho subfamily proteins such as Rho, Rac, and Cdc 42. These proteins mainly play an

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important role in the actin cytoskeleton dynamic system. They also influence cell-cell contact, integrin signaling, endocytosis, transcriptional activation, proliferation, apoptosis, and cell transformation [5]. TcdA and TcdB increase paracellular permeability by the disorganization of apical and basal F-actin and the dissociation of occludin, ZO-1, and ZO-2 from the lateral tight junction membrane [6]. In a human intestinal cell line, TcdA has been shown to cause apoptosis in a dose- and time-dependent fashion by mechanisms both dependent and independent on inactivation of Rho; activation of caspases 3, 6, 8 and 9; cleavage of Bid; and mitochondrial damage followed by cytochrome C release [7, 8]. TcdB also has been shown to cause apoptosis in a dose- and time-dependent manner [9]. Similarly, both caspase-dependent and -independent pathways are involved, as with TcdA-intoxicated cells [10]. The clinical relevance of CDT is yet to be proven, although its role in colonization and bacterial adherence by the formation of microtubule-based protrusions has been implicated [11].

Glutamine is a major fuel or substrate for enterocytes and cells of the immune system. Glutamine uptake and metabolism are increased in glutamine-using tissues, such as the intestinal mucosa during stress. In animal models of intestinal atrophy, injury, and adaptation, glutamine-supplemented parenteral and enteral nutrition enhances gut mucosal growth, repair, and function; decreases gut-associated sepsis; and improves nitrogen balance [12, 13]. Indeed, it has been previously shown that glutamine and alanyl-glutamine (AQ) reduced the damage caused by TcdA in T84 cells, by inhibiting caspase 8 activation, thereby preventing apoptosis; in rabbit ileal loops, by decreasing inflammation and secretion [8, 19]; and in IEC6 cells, by reducing apoptosis and enhancing migration [14]. Because all known clinical isolates of *C. difficile* carry TcdB but not necessarily TcdA, we evaluated in this study whether AQ can likewise ameliorate the effects of the more potent cytotoxin, TcdB, in intestinal cells and, moreover, whether AQ can improve outcomes of *C. difficile* infection in mice.

METHODS

Proliferation Assay

A rat small intestinal epithelial crypt cell line (IEC-6, passage 17) was obtained from ATCC. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (Gibco), 1 mM sodium pyruvate, 95% bovine insulin, and 50 U/mL Pen/Strep, maintained in a humidified incubator at 37°C and 10% CO_2. IEC-6 cells in glutamine-free DMEM (Gibco) were seeded in 96-well plates at a concentration of 10^5 cells/well and allowed to grow until 80% confluence. Then, the cells were challenged with 100 μL of TcdB of varying concentrations (0.001–1 ng/mL) or TcdA (10 ng/mL), with or without AQ (Sigma, St. Louis, MO), in concentrations of 10 mM, 1 mM, and 0.1 mM diluted in DMEM glutamine-free medium. Nontreated IEC-6 cells grown in glutamine-free or glutamine-enriched medium served as controls. Cell proliferation was measured by the use of WST-1 (Roche, Indianapolis, IN), a tetrazolium salt that, in the presence of mitochondrial dehydrogenase, is cleaved to formazan. The increase in formazan is observed by light absorbance. After 24 and 48 hours, 100 μL of the cell-proliferation reagent WST-1 was added into the well plates to measure the metabolic activity of viable cells. Cells were incubated with WST-1 for 1 hour, during which viable cells converted WST-1 to a water-soluble formazan dye. Absorbances were measured at 450 nm, using an enzyme-linked immunosorbent assay plate reader (BioTek Instruments). The absorbance directly correlates with cell number.

Migration Assay

IEC-6 cells (passage 19) were used for this wounding assay. IEC-6 cells were seeded in 6-well plates at a concentration of 10^6 cells/well and were allowed to grow until total confluence. The confluent cells were scratched with a razor blade at the midline, extending to the right side of the well. The intention of the scrape is to simulate epithelial damage and measure cell migration from the injured site during the early phases of recovery in the tissue [15]. After the scrape or "wounding," the wells were washed 2 times with phosphate-buffered saline (PBS), and the medium was changed to glutamine-free medium containing TcdB (0.001–1 ng/mL). Another set of experiments was performed, in which some TcdB-challenged cells remained untreated and others were treated with AQ in concentrations of 10 mM, 1 mM, or 0.1 mM diluted in glutamine-free DMEM. Migrated cells were observed under 10× magnification after 24 and 48 hours, using an Olympus 1 × 71 inverted microscope with a QImaging camera. Images were taken using QCapture Pro.5.1 software, and cells were counted per measured area, expressed in millimeters squared.

Apoptosis Assay

Apoptosis and necrosis were assessed by flow cytometry. The Annexin V kit (BD Pharmingen) was used to detect phosphatidylserine on the reverse membrane surface of apoptotic cells. Propidium iodide was used to detect nucleic acids inside necrotic and late apoptotic cells. IEC-6 cells were seeded in 6-well plates at a concentration of 10^6 cells/well and incubated for 24 hours. Then, the cells were treated with TcdB (0.01 ng/mL), with or without AQ (10 mM), and incubated for another 24 hours. The medium was changed after exposure, and cells were washed twice with PBS, trypsinized, collected by centrifugation, and double stained with fluorescein isothiocyanate–conjugated Annexin V and propidium iodide. The samples were then processed at the University of Virginia Flow Cytometry Core, using a BD FACSCalibur dual laser.

Murine Model of *C. difficile* Infection

The infection model is a modification of the protocol published by Chen et al [16]. This protocol has been approved by the Center for Comparative Medicine at the University of Virginia. Male C57BL/6 mice aged 8 weeks were used. During the 4–6-day period before infection, mice were given an antibiotic cocktail containing vancomycin (0.0045 mg/g), colistin (0.0042 mg/g), gentamicin (0.0035 mg/g), and metronidazole (0.0215 mg/g) per orem. One day prior to infection, clindamycin (32 mg/kg) was injected subcutaneously. All antibiotics were obtained from the University of Virginia Hospital Pharmacy. The mice were divided into the following groups: control uninfected, control infected, infected treated with AQ (100 mM in the drinking water), infected treated with vancomycin (50 mg/kg) alone, and infected treated with vancomycin (50 mg/kg) and AQ (100 mM). Food and water were given ad libitum to the mice. Infection was performed with VPI 10463 (ATCC) at an inoculum of 10^4–10^5 by oral gavage. One day after infection, infected treated mice received AQ alone for 14 days or vancomycin (50 mg/kg) daily for 5 days, with or without AQ (in drinking water) for 14 days, with the first 5 days overlapping vancomycin receipt. Daily weights were recorded, and mice were monitored for diarrhea and activity level. Diarrhea was scored as previously described [17]. Moribund mice at any day of the experiment and all surviving mice at day 14 were euthanized.

Histopathologic Analysis

Cecal and colonic tissues were harvested and fixed in 10% zinc formalin. Hematoxylin-eosin staining was performed at the University of Virginia School of Medicine Research Histology Core for histopathology. Tissues were also processed for immunohistochemistry analysis (DAKO Autostainer) to detect mouse poly(ADP-ribose) polymerase 1 (PARP-1), an enzyme proteolyzed by caspases during apoptosis, and mouse myeloperoxidase (MPO), expressed by inflammatory cells, using anti-rabbit polymer (DAKO Envision; UVA Biorepository and Tissue Research Facility). Antibodies for PARP-1 and MPO were from Invitrogen and Nuvus Biologicals, respectively. Histopathologic findings were scored on the basis of cellularity, mucosal disruption, mucosal hypertrophy, exudate, and submucosa edema, as we previously reported [18]. Apoptotic cells were counted in at least 30 random high-power fields in each tissue section. Representative tissue sections from each treatment group were photographed.

Statistical Analysis

Results were expressed as mean values ± standard error of the mean (SEM), as generated by GraphPad Prism, version 5.0 (GraphPad, San Diego, Ca). The differences between experimental groups were compared using analysis of variance with the Bonferroni multiple comparison test. Survival curves were subjected to the log-rank (Mantel-Cox) test. Statistical significance was set at $P < .05$.

RESULTS

Effect of AQ on TcdB-Induced Inhibition of Cell Proliferation

Because intestinal cell restitution after injury involves both cell proliferation and migration, we studied the effect of TcdB on each function. In the proliferation assay, TcdB given at incremental doses (0.001, 0.01, 0.1, and 1 ng/mL) impaired IEC-6 proliferation in a dose-dependent fashion. IEC-6 cells challenged with TcdB at 0.01 ng/mL and treated with AQ showed significant improvement of proliferation in a dose-dependent manner after 24 and 48 hours of incubation (Figure 1). Cells exposed to TcdB at a dose of 0.1 ng/mL did not respond to AQ at any of the concentrations. TcdB at a dose of 0.001 ng/mL did not significantly decrease IEC-6 cell proliferation, while a 1-ng/mL dose resulted in complete detachment of all cells in the treated wells.

Effect of AQ on TcdB-Induced Inhibition of Enterocyte Migration

To confirm the beneficial effect of AQ on TcdB-induced epithelial damage, we treated TcdB-challenged IEC-6 cells in the cell-wounding assay. TcdB inhibited IEC-6 migration in a dose-dependent fashion, both at 24 and 48 hours. TcdB (0.001 ng/mL) impaired cell migration by 61% at 24 hours. At a dose of 1 ng/mL, cell migration was completely inhibited. TcdB at a dose of 0.01 ng/mL was chosen to study the effect of AQ, on the basis of results of the proliferation study above. AQ enhanced migration in unchallenged cells (Figure 2). As observed in the proliferation assay, AQ increased migration in TcdB-challenged cells in a dose-dependent manner, with the highest dose of AQ (10 mM) showing a significant increase in migration 24 and 48 hours after wounding.

Effect of AQ on TcdB-Induced Apoptosis and Necrosis

Flow cytometry–based quantification of IEC-6 cells double stained with Annexin V and propidium iodide and challenged with TcdB (0.01 ng/mL) alone revealed that 27% of cells were apoptotic and 8% were necrotic (Figure 3). After TcdB challenge, the number of apoptotic cells increased by 3-fold, compared with the value for controls, whereas the number of necrotic cells was not significantly different from that for controls. Concomitant treatment with AQ (10 mM) for 24 hours significantly reduced apoptosis and necrosis to 8% and 4% of cells, respectively.

Effect of AQ on *C. difficile*–Infected Mice

C57BL/6 mice infected with VPI 10463 at an inoculum of 10^5 developed diarrhea and progressively lost weight as compared to uninfected mice. The lowest weights and the highest diarrhea scores for the infected control mice were noted around

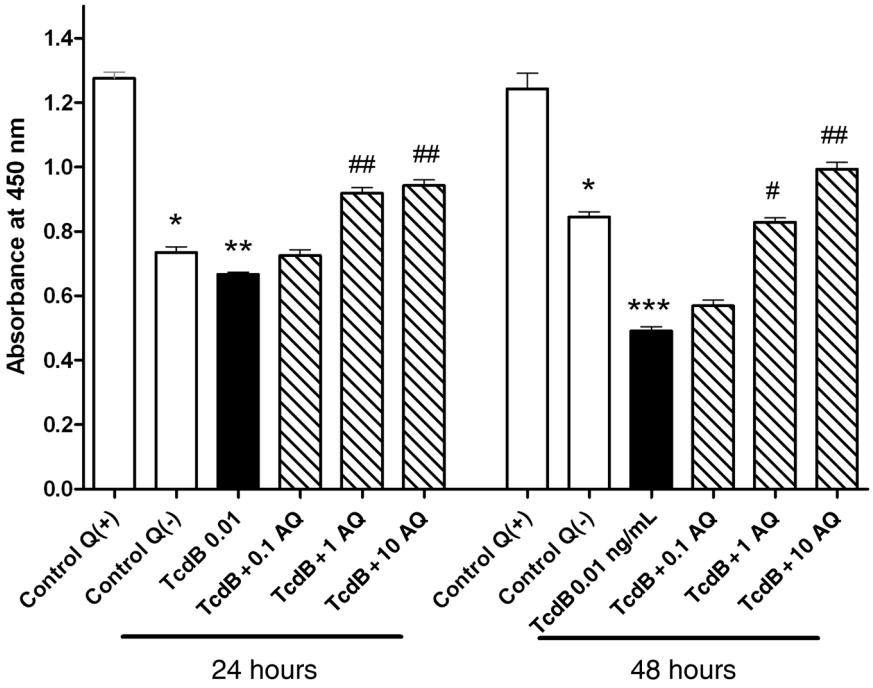

Figure 1. Effect of alanyl-glutamine (AQ) on *Clostridium difficile* toxin B (TcdB)–challenged IEC-6 cell proliferation. IEC-6 cells were grown in glutamine (Q)–deficient medium and challenged with TcdB at 0.01 ng/mL, with or without AQ (concentration, 0.1, 1, or 10 mM). Optical density readings were performed 24 and 48 hours after incubation. All groups had at least 7 wells. $^{*}P < .001$ vs Q+ control, $^{**}P < .05$ vs Q– control, $^{***}P < .001$ vs Q– control, $^{\#}P < .001$ vs TcdB, and $^{\#\#}P < .001$ vs TcdB or Q– control, by analysis of variance with the post-hoc Bonferroni multiple comparison test.

days 3–4 and days 3–7 after infection, respectively (Figure 4*C* and 4*E*). Survival among untreated infected mice was 15% (2 of 13) by day 6; the majority of mice (8 of 11) died of infection at day 4 (Figure 4*A*). Likewise, infected mice treated with AQ alone had diarrhea and weight loss (data not shown); 11 of 14 (79%) died from infection by day 6, with the majority of deaths (7 of 11) also occurring on day 4. Infected mice treated with vancomycin for 5 days exhibited minimal decreases in weights and maintained 100% survival until 3 days after completion of treatment, when deaths started to occur. Only 2 of 14 infected mice (14%) treated with vancomycin were alive by day 13. In contrast, 6 of 14 infected mice (43%) treated with both vancomycin (for 5 days) and AQ (for 14 days) survived.

To induce a milder form of infection, we also infected mice with only 10^4 of VPI 10463. Infected mice (n = 8) developed diarrhea between days 2 and 6 but, like uninfected controls, did not die of infection (Figure 4*B*, 4*D*, and 4*F*). Similarly, all mice (n = 8) treated with both vancomycin and AQ had delayed onset of diarrhea (on days 10–13), but all survived infection. As observed in high-dose infection (10^5) described above, the group of infected mice treated with vancomycin developed mild diarrhea, and deaths in the group did not begin to occur until a few days after treatment. In this group, 3 of 8 mice (37.5%) eventually died. Of 8 infected mice that did not receive vancomycin but were given AQ, only 1 mouse died. These findings indicated that AQ supplementation improves the disease manifestation and outcome of both severe and mild *C. difficile* infection in mice.

Effect of AQ on *C. difficile*–Induced Intestinal Histopathologic Abnormalities, Inflammation, and Apoptosis

Intestinal tissues were harvested from euthanized moribund mice, usually on days 3–6 for untreated (or treated but not responding to treatment) infected mice and day ≥8 for vancomycin-treated infected mice. Histopathologic study of cecal tissues revealed mucosal disruption, inflammatory cell infiltration, exudates, mucosal hypertrophy, and submucosal edema in infected mice, with a mean histopathology score (±SEM) of 11.7 ± 1.4, compared with 1 ± 0.4 for uninfected controls (Figure 5). Colonic tissues followed a similar trend. Histopathologic analysis of euthanized moribund mice in the vancomycin-treated group showed elevated scores that were nearly as high as those of the infected controls at the time of death (Figure 5*C*). Mice treated with both vancomycin and AQ showed improved mean histopathology scores (±SEM) of 4.8 ± 1.3. Inflammation, as assessed by staining for myeloperoxidase activity, also showed improvement with combination treatment (Figure 5). Moreover, PARP-1 staining revealed significant numbers of apoptotic cells in intestinal tissues from infected mice and a decrease in the number of apoptotic cells

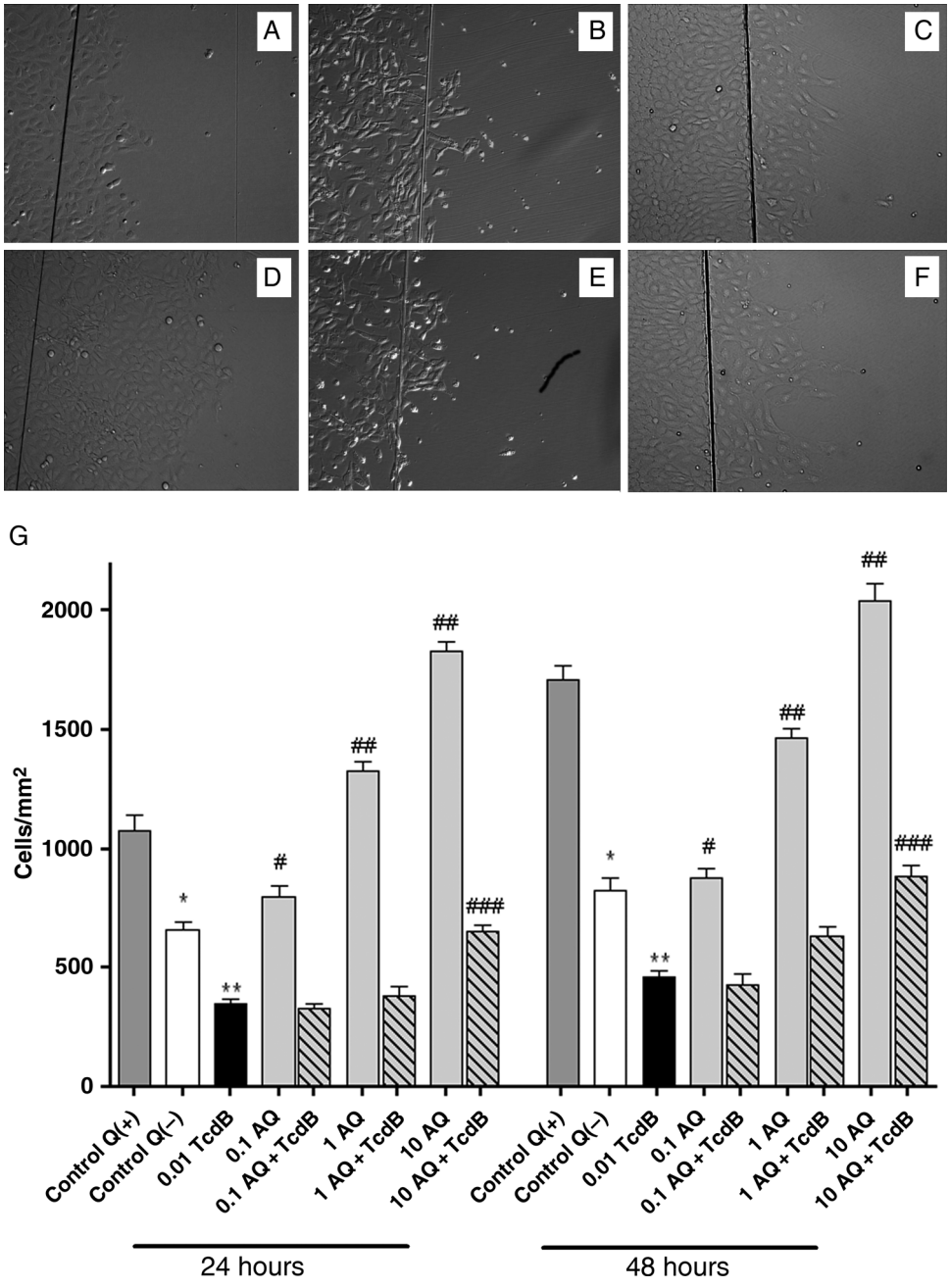

Figure 2. Effect of alanyl-glutamine (AQ) on *Clostridium difficile* toxin B (TcdB)–challenged IEC-6 cell migration in a wounding assay. IEC-6 cells were grown in glutamine (Q)–deficient media with or without TcdB at 0.01 ng/mL, with or without AQ treatment (concentration 0.1, 1, and 10 mM). Migrating cells were counted 24 and 48 hours after wounding. Representative wells of control cells (*A* and *D*), TcdB-treated cells (*B* and *E*), and TcdA and AQ (10 mM)–treated cells (*C* and *F*) cells 24 (*A*–*C*) and 48 (*D*–*E*) hours after wounding are shown. *G*, Each bar represents the mean number of migrating cells per treatment group. Each treatment group had 4 wells. $^{*}P < .001$ vs Q+ control, $^{**}P < .001$ vs Q– control, $^{\#}P < .001$ vs TcdB + AQ, $^{\#\#}P < .001$ vs TcdB or Q– control, and $^{\#\#\#}P < .001$ vs TcdB, by analysis of variance with the post-hoc Bonferroni multiple comparison test.

in infected mice treated with vancomycin plus AQ (Figure 6). Together, these findings suggest that vancomycin-treated mice are susceptible to further intestinal tissue injury from relapse of infection and that concomitant administration of AQ reduces the late-onset histopathologic abnormalities after antibiotic treatment.

DISCUSSION

The results of this study confirm that *C. difficile* toxins inhibit proliferation and migration and induce apoptosis in intestinal epithelial cells. Prior studies have shown that AQ reduces these TcdA-induced effects in intestinal epithelial cells

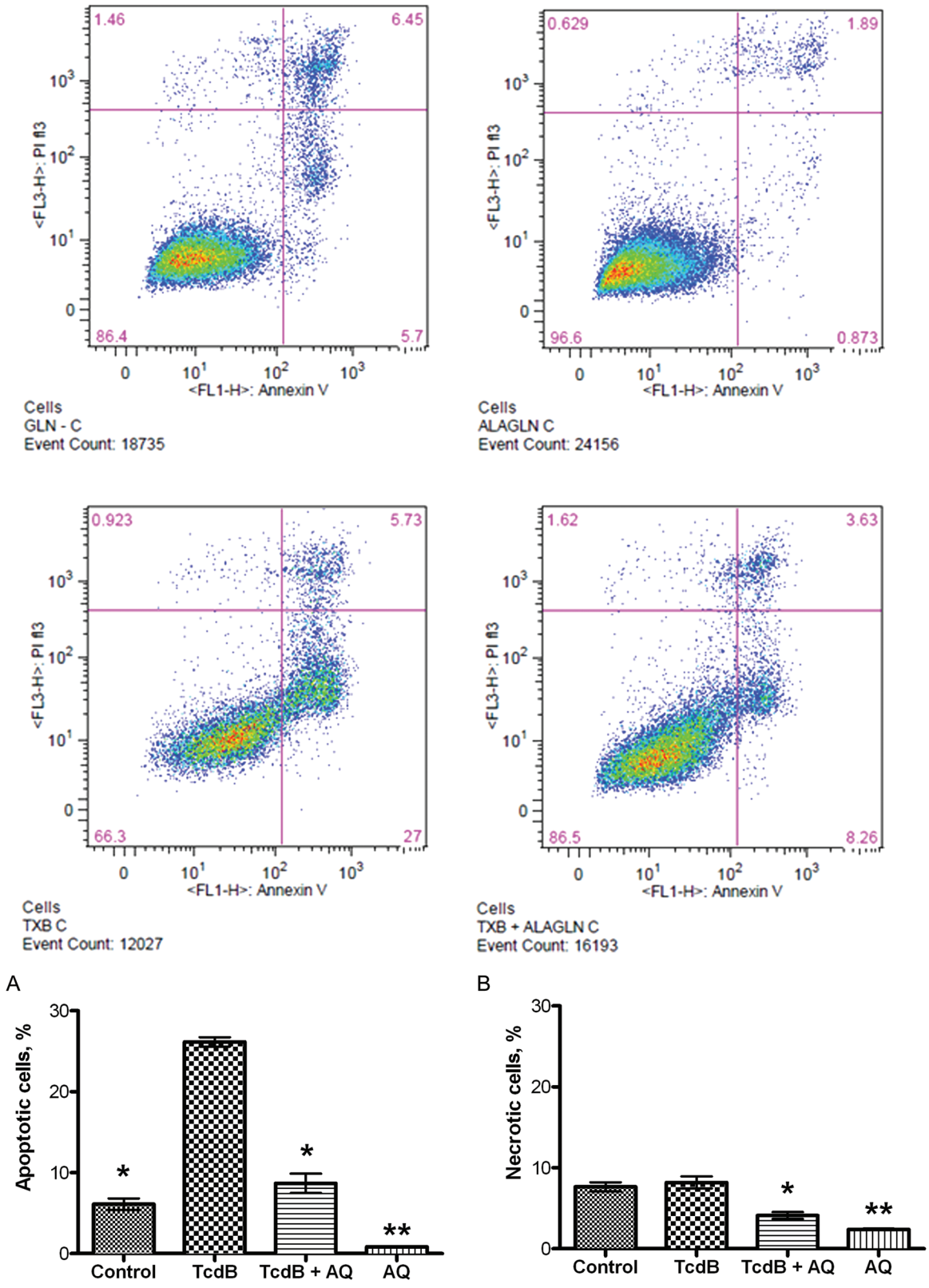

Figure 3. Flow cytometry density plots of propidium iodide vs annexin V–fluorescein isothiocyanate in IEC6 cells treated with control (glutamine free; GLN-C), alanyl-glutamine (AQ/ALAGLN, 10 mM) alone, toxin B (TcdB/TXB, 0.01 ng/mL), and TcdB + AQ (TXB + ALAGLN). Number of apoptotic and necrotic cells are represented in the bar graphs as the percentage of the total number of cells counted per sample. Each treatment group represents 3 samples read by flow cytometry. *A*, **P* < .001 for TcdB vs control or TcdB + AQ, ***P* < .01 for control vs AQ. *B*, **P* < .01 for TcdB vs TcdB + AQ and ***P* < .001 for control vs AQ alone. All comparisons were performed by analysis of variance with the Bonferroni multiple comparison test.

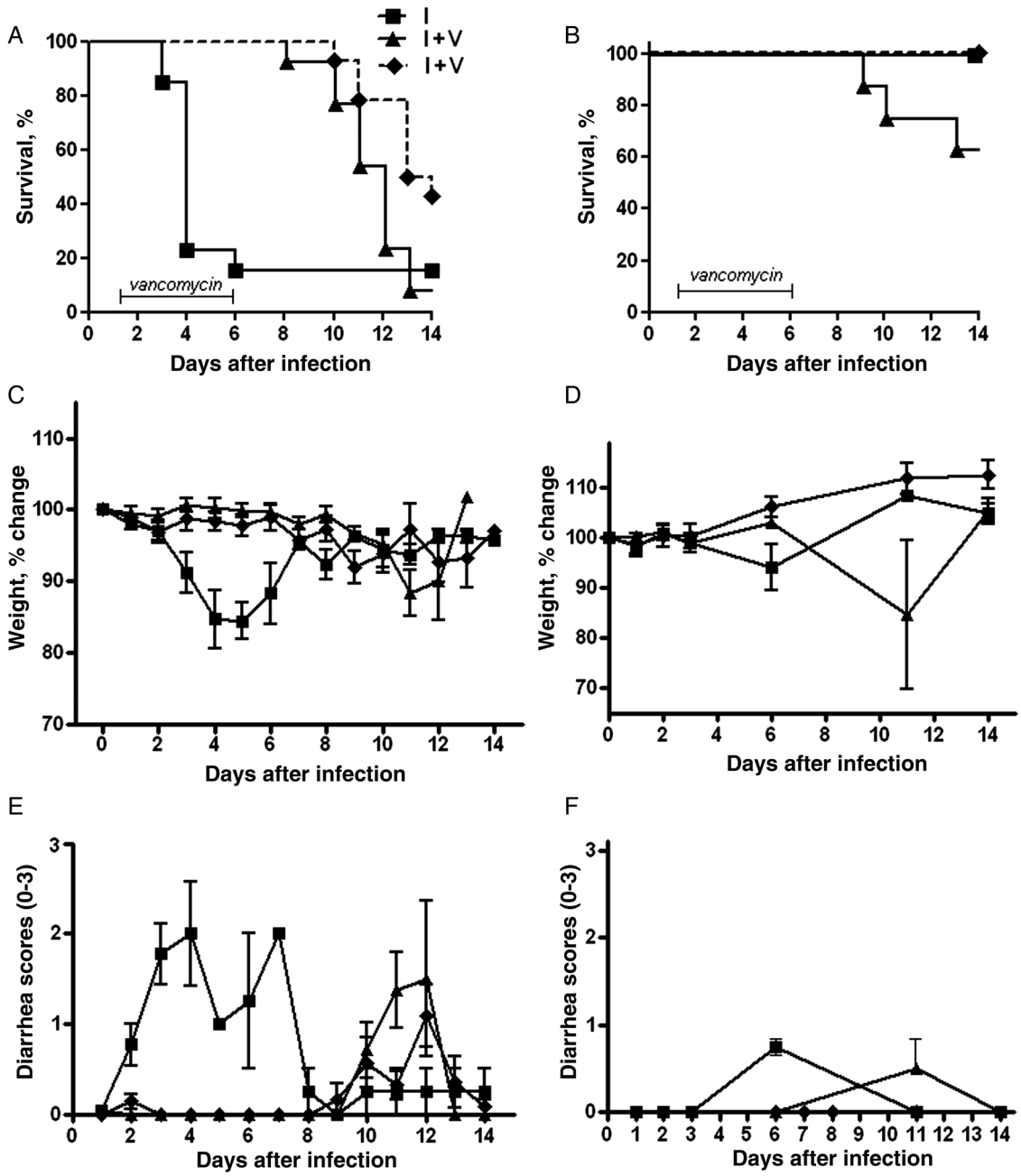

Figure 4. Effects of alanyl-glutamine (AQ) supplementation in *Clostridium difficile*–infected mice. *A, C,* and *E,* Mice were infected with 10^5 VPI10463. In treated mice, vancomycin (50 mg/kg/day) was given from days 1–5 and AQ (100 mM) was given from days 1–14. All groups had 14 mice, except for the control group (n = 7) and the infected group (n = 13). Survival curve: $P < .01$ for infected mice treated with vancomycin (I + V) vs infected mice treated with both vancomycin and AQ (I + VAQ), by the Mantel-Cox test. *B, D,* and *F,* Mice were infected with 10^4 VPI10463. Similar treatments were given as in panel *A*. All groups had 8 mice each, except for the control group (n = 4). Survival curve: $P = .06$ for I + V vs I + VAQ, by the Mantel-Cox test. The diarrhea scoring system ranged from 0 (mild) to 3 (severe).

in vitro [7, 8, 14] and in intestinal tissues of mice and rabbits [8, 19]. For the first time, we demonstrate that AQ is similarly effective in reversing TcdB effects in enterocytes. Most importantly, we now also show evidence that AQ supplementation may be beneficial in *C. difficile* infection. In the murine model of *C. difficile* infection, AQ, when given with vancomycin, improved diarrhea scores, weights, and survival rates. Similar to observation for epithelial cells, the

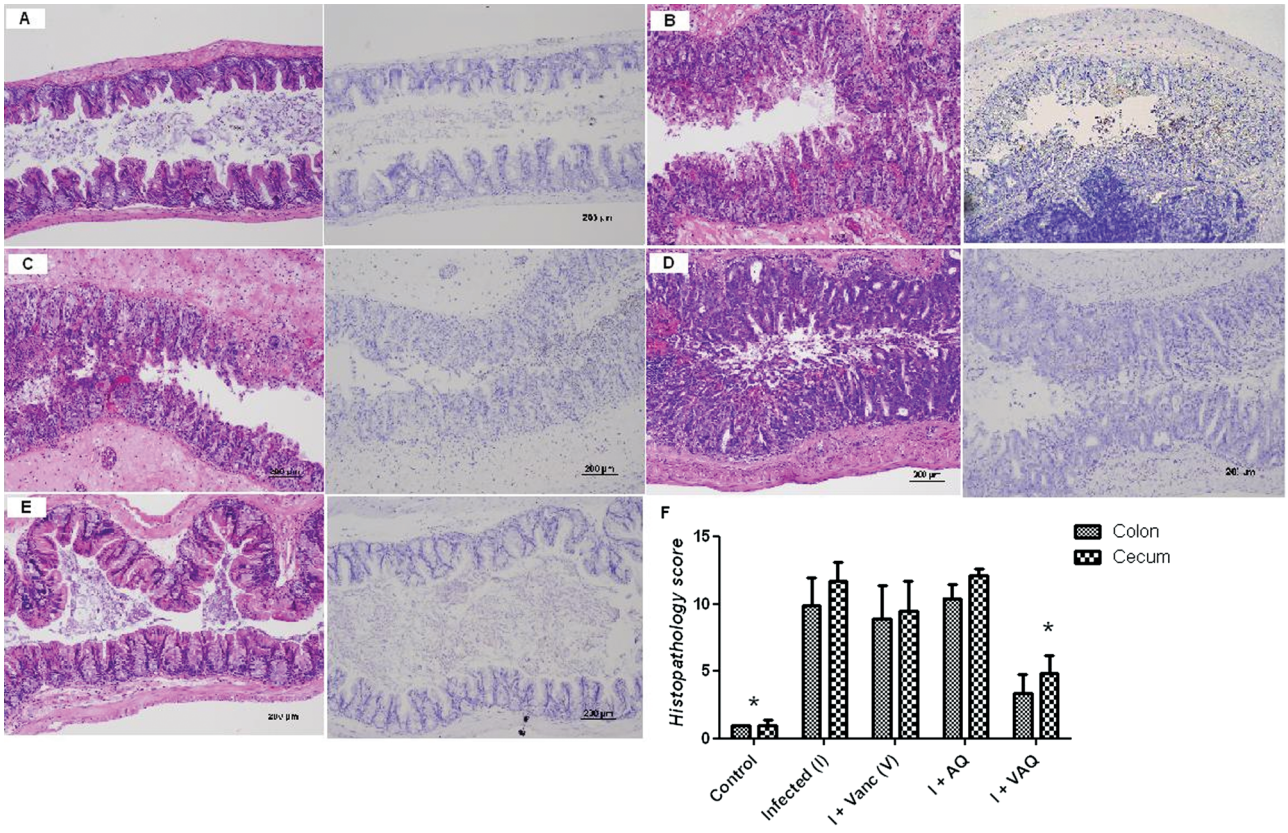

Figure 5. Intestinal tissues from *Clostridium difficile*–infected mice stained with hematoxylin-eosin (columns 1 and 3) and myeloperoxidase (columns 2 and 4). Shown is tissue from a control mouse (*A*), an infected mouse (I; *B*), an infected mouse treated with vancomycin (I + V; *C*), an infected mouse treated with alanyl-glutamine (I + AQ; *D*), and an infected mouse treated with both vancomycin and AQ (I + VAQ; *E*). Histopathology scores (*F*) range from 0 (normal) to 15 (worst). See Methods and references for a description of the scoring system. Findings for infected mice that were found dead were scored as 15. There were 3 control mice, 5 infected mice, 6 mice per treatment group. $^{*}P < .01$ for I vs control (both tissues) and $^{*}P < .05$ for I vs I + VAQ (cecum), by 1-way analysis of variance with the Bonferroni multiple comparison test.

intestinal epithelium in infected mice treated with both vancomycin and AQ had improved histopathologic findings and decreased apoptosis. Moreover, inflammation, as observed with MPO staining, was reduced.

When damaged, the gastrointestinal mucosa has the ability to repair itself very rapidly, almost entirely in 24 hours [20]. The early phase of repair is termed mucosal restitution and involves the sloughing of damaged epithelial cells and migration of remaining viable cells to reestablish epithelial continuity [21]. Migration occurs independent of cell division on the first hours after injury [15]. Because infection may be caused by TcdA-negative but TcdB-positive bacteria, we prove that TcdB alone can also prevent proliferation and migration of intestinal epithelial cells in vitro. Both *C. difficile* toxins cause glucosylation of Rho proteins, which are important to spontaneous movement of intestinal cells (by cytoskeleton activity) and EGF-induced migration [22]. Inhibition of Rho functions may delay the early, in addition to the late, phase of the mucosal healing process. *C. difficile* toxins activate proapoptosis pathways [7–9, 23], facilitating cell death and further decreasing the pool of regenerative cells for repair, as we have seen here in vitro and in vivo.

AQ has been an attractive source of free glutamine because of its stability, safety, and excellent solubility [24, 25]. In this study, AQ significantly decreased TcdB-induced inhibition of proliferation, migration, and apoptosis among IEC 6 cells. Glutamine increases expression of proliferating cell nuclear antigen (PCNA), which is involved in the DNA synthesis phase of cell cycle and repair during DNA damage [26], but this activity has not been observed for AQ. PCNA is underexpressed during injury, such as that caused by exposure to chemotherapy [26]. Indeed, we have previously shown that AQ reversed epithelial damage caused by 5-fluorouracil [27]. In T84 cells, TcdA causes apoptosis, by activation of caspases 3, 6, 8, and 9 and cleavage of Bid, and mitochondrial damage [7, 8]. Glutamine and AQ protected against apoptosis by inhibiting activation of caspase 8 and reduced toxin-induced intestinal secretion and mucosal disruption in rabbit

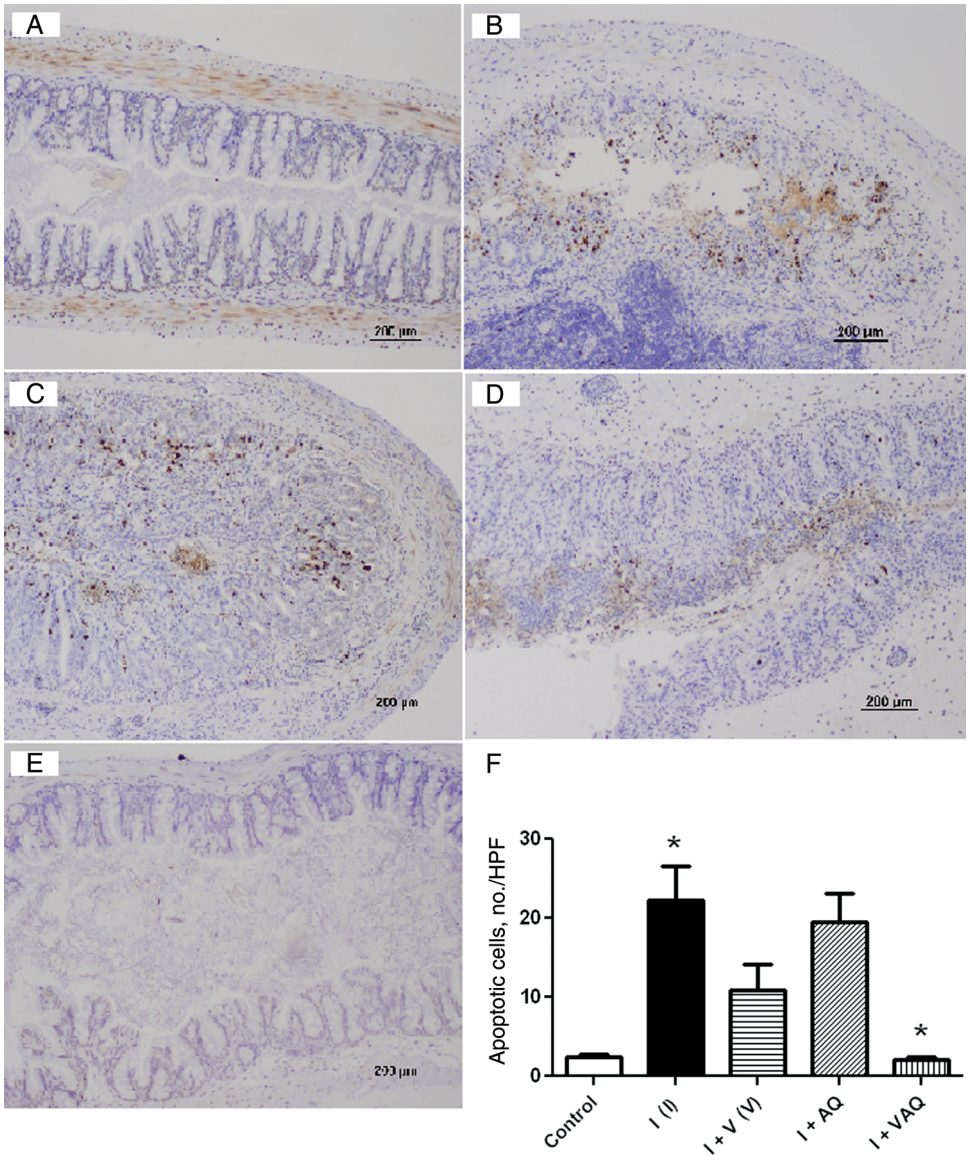

Figure 6. Representative mouse cecal tissues stained for poly(ADP-ribose) polymerase 1 (PARP-1) as a marker of apoptotic cells (brown). Shown is tissue from a control mouse (*A*), a *Clostridium difficile*–infected mouse (I; *B*), an infected mouse treated with vancomycin (I + V; *C*), an infected mouse treated with alanyl-glutamine (I + AQ; *D*), and an infected mouse treated with both vancomycin and AQ (I + VAQ; *E*). *F*, Numbers of apoptotic cells in each group (30 high-power fields [HPFs] reviewed per slide; 2 representative slides per group). **P* < .001 for I vs control or I + VAQ, by 1-way analysis of variance with the Bonferroni multiple comparison test.

ileal tissues [8]. TcdB has also been shown to cause apoptosis by caspase-dependent and -independent pathways [10]. In addition to induction of apoptotic pathways, glutamine may be involved in the activation of ERK, an important signaling pathway for DNA synthesis, cell proliferation, and antiapoptosis in numerous cell lines [28].

Because we found that AQ reversed TcdB effects in vitro, we hypothesized that AQ may improve outcomes of *C. difficile* infection. In the murine model of *C. difficile* infection, C57BL/6 mice that were infected but untreated and those that were infected but treated only with AQ had similar mortality rates, suggesting the need for antimicrobial treatment to treat the disease. However, infected mice treated with vancomycin did well until 3 days after discontinuation of treatment, when the animals started dying, with the final outcome similar to if not worse than that for untreated infected mice. This delay in mortality associated with vancomycin treatment suggests that, although antimicrobial therapy may be beneficial during acute

infection, it may not be adequate to prevent recurrence of disease and death, as we and others have previously observed [16, 17, 29]. The exact mechanism underlying vancomycin-associated relapse is unclear, but alteration in intestinal microbiota may be involved [30]. While vancomycin was shown to be effective in reducing vegetative forms and cytotoxin levels of 2 epidemic *C. difficile* strains in a human gut model, it showed no activity against spores [31]. An altered gut flora may facilitate germination of either residual spores (relapse) or spores acquired from elsewhere (reinfection). Interestingly, infected mice that received both vancomycin and AQ had improved survival, associated with intestinal tissues revealing less histopathologic abnormalities, inflammatory cell infiltration, and apoptosis, suggesting that AQ may be able to limit or prevent intestinal epithelial damage from either initial infection or relapse.

Glutamine, from the breakdown of AQ, is the main fuel for enterocytes and is essential in the recovery of damaged epithelium and tight junctions and avoidance of bacterial translocation [6]. Indeed, histopathologic abnormalities, myeloperoxidase activity, and apoptotic intestinal cells were reduced in mice treated with both vancomycin and AQ in this study. Although the role of AQ in the systemic inflammatory response against toxins or pathogens was not assessed in this study, AQ may also play a regulatory role in this response, as observed in other conditions [32–34]. Moreover, AQ may have prevented death through water loss (during diarrhea) by activating Na and water absorption [35, 36]. Since diet has been shown to affect the intestinal microbiota [37, 38], it is also possible that AQ may have a role in their preservation or enhanced recovery after antimicrobial therapy, preventing overgrowth of toxigenic *C. difficile*. Whether other dipeptides, including glutamine-free dipeptides, provide similar benefits during *C. difficile* infection was not addressed by our study and warrants further investigations.

In summary, this study demonstrates that AQ may ameliorate *C. difficile* toxin–induced intestinal cell injury by enhancing epithelial repair, reducing inflammation, and preventing cell death, thereby decreasing intestinal tissue damage, reducing susceptibility to recurrent disease, and improving survival among infected mice after antimicrobial treatment. AQ supplementation may play an adjunctive role in the antibiotic treatment of *C. difficile* infection.

Notes

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Potential conflict of interest. R. L. G cofounded AlGlutamine. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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