Human Monoclonal Antibodies against *Clostridium difficile* Toxins A and B Inhibit Inflammatory and Histologic Responses to the Toxins in Human Colon and Peripheral Blood Monocytes

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*Clostridium difficile* infection (CDI) is a common and debilitating nosocomial infection with high morbidity and mortality. *C. difficile* mediates diarrhea and colitis by releasing two toxins, toxin A and toxin B. Since both toxins stimulate proinflammatory signaling pathways in human colonicocytes and both are involved in the pathophysiology of CDI, neutralization of toxin A and B activities may represent an important therapeutic approach against CDI. Recent studies indicated that human monoclonal antibodies (MAbs) against toxins A and B reduce their cytotoxic and secretory activities and prevent CDI in hamsters. Moreover, anti-toxin A and anti-toxin B MAbs together with antibiotics also effectively reduced recurrent CDI in humans. However, whether these MAbs neutralize toxin A- and toxin B-associated immune responses in human colonic mucosa or human peripheral blood monocyte cells (PBMCs) has never been examined. We used fresh human colonic biopsy specimens and peripheral blood monocytes to evaluate the effects of these antibodies against toxin A- and B-associated cytokine release, proinflammatory signaling, and histologic damage. Incubation of anti-toxin A (MK3415) or anti-toxin B (MK6072) MAbs with human PBMCs significantly inhibited toxin A- and toxin B-mediated tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) expression. MK3415 and MK6072 also diminished toxin A- and toxin B-mediated NF-κB p65 phosphorylation in human monocytes, respectively, and significantly reduced toxin A- and B-induced TNF-α and IL-1β expression as well as histologic damage in human colonic explants. Our results underline the effectiveness of MK3415 and MK6072 in blocking *C. difficile* toxin A- and toxin B-mediated inflammatory responses and histologic damage.

*Clostridium difficile* infection (CDI) is a common gastrointestinal nosocomial infection with increasing incidence and mortality over the past decade (1). The pathophysiology of CDI involves the release of two large exotoxins from pathogenic strains, toxin A and toxin B (2). Despite early reports indicating that toxin A is the primary toxin mediating the enterotoxic effects of *C. difficile*, studies in human colonic explants and human colonic xenografts indicate that both toxins can induce inflammation and cytotoxicity in human colon (3, 4). Studies in hamsters using isogenic toxin A and toxin B mutants of a virulent *C. difficile* strain confirmed that both toxins are important for the pathophysiology of CDI (5, 6).

The proinflammatory action of toxins A and B involves release of various cytokines and chemokines, prostaglandins, and additional inflammatory mediators from human colonicocytes (7, 8) and monocytes (9, 10). The mechanisms involved in the toxin actions involve activation of established proinflammatory signaling pathways, including the global mediator of inflammation NF-κB (7) (11, 12), mitogen-activated protein (MAP) kinases (10, 13), as well as cyclooxygenase-2 (14).

Earlier evidence indicated that immune responses to *C. difficile* toxins are an important component for the pathogenesis of CDI (15–17). More recent studies confirmed the importance of anti-toxin antibodies against toxin A and toxin B in primary and recurrent CDI in humans (18, 19) and animals (20, 21). Human monoclonal antibodies (MAbs) against toxin A reduced mortality in hamsters caused by *C. difficile*, but neutralization of both toxins provided better protection (22). Both monoclonal antibodies also neutralized the cytotoxicity of both toxins in human fibroblasts (22). Importantly, the results of a large multicenter phase II trial using human neutralizing MAbs against both toxins, together with standard antimicrobial therapy, in CDI patients showed a significant reduction of recurrent CDI (23).

Although the efficacy of human MAbs against toxins A and B in reducing relapsing CDI has been demonstrated (23), formal studies on the effect of these antibodies in the toxin A- and B-mediated innate immune responses in target human cells or human colonic mucosa *in vitro* have not been done. In this study, we addressed the hypothesis that neutralization of toxins A and B by human MAbs against toxin A (MK3415) or toxin B (MK6072) provided by Merck inhibits toxin A- and B-mediated innate immune responses in human colonic mucosa and human peripheral blood...
monocyte cells (PBMCs). Our results demonstrate that both MAbs can significantly reduce toxin A- and toxin B-mediated expression of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in monocytes and human colonic mucosal biopsy specimens and diminish epithelial tissue damage in human colonic tissues in response to these toxins.

MATERIALS AND METHODS

C. difficile culture and toxin purification. C. difficile strain VPI 10463 (ATCC 43255 stock strain) was cultured in Difco cooked meat medium (catalog no. 226730; BD, Fisher Scientific) at 37°C under anaerobic conditions, and toxins A and B were purified to homogeneity as previously reported (3). The cytotoxicity of toxins A and B was determined by cell rounding, as previously described (24).

Human colonic explants and human primary monocyte cell culture. Colonic explants and blood were obtained after informed consent in accordace with procedures established by the Cedars-Sinai Institutional Review Board (number 3358). PBMCs were isolated as we described previously (25). Human primary monocytes were isolated and cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen) as described previously (25). Monocyte preparations were 90% pure, as determined by esterase stain (Sigma-Aldrich, St. Louis, MO).

Mouse macrophage cell culture. Mouse cultured RAW264.7 macrophages were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen), as previously described (25).

Merck MK3415 and MK6072 neutralizing antibodies. Anti-toxin A MK3415 and anti-toxin B MK6072 neutralizing antibodies were provided by Merck and used at the indicated concentrations. Details on the generation, characterization, and in vitro and in vivo neutralizing activities of these monoclonal antibodies were described by Babcock et al. (22). MK3415 was formerly called MDX006, and MK6072 was formerly called MDX1388. Control human IgG antibody was obtained from Southern Biotech (catalog no. 0150.01).

TNF-α and IL-1β ELISAs. The levels of proinflammatory mediators human TNF-α (DY210, R&D Systems) and human IL-1β (DY201; R&D Systems) were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions.

FIG 1 MK3415 attenuated toxin A-mediated TNF-α expression in PBMCs. (A and B) PBMCs were incubated with no antibody (blank), control IgG, or MK3415 (30 µg/ml) for 30 min, followed by incubation with toxin A at 0 to 1 µg/ml for 4 h. (C and D) PBMCs were incubated with control IgG or MK3415 (0.1 to 50 mg/ml) for 30 min, followed by incubation with toxin A at 0.1 µg/ml for 4 h. Conditioned media were collected for TNF-α ELISA. TNF-α mRNA expression was detected by real-time RT-PCR. All experiments are representative of 3 independent experiments.
Phospho-NF-κB p65 and phospho-ERK ELISAs. The treated cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitor cocktail. Equal amounts of proteins (∼40 μg) were used for detection of phospho-p65 (kit number 7834; Cell Signaling, Danvers, MA) and phospho-extracellular signal-regulated kinase (phospho-ERK; kit number 7246; Cell Signaling) using ELISAs, according to the manufacturer’s instructions.

Western blot analyses. Cells were lysed in 1X blue loading buffer (catalog no. 7722; Cell Signaling, Danvers, MA). Equal amounts of cell extracts were fractioned by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membranes (400 mA for 2 h at 4°C; Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and then incubated with antibodies (catalog no. 4370 for phospho-pERK and catalog no. 2128 for -tubulin; Cell Signaling). Horseradish peroxidase-labeled antibody labeling was detected by chemiluminescence using an LAS4000 luminescent image analyzer (Fuji-film, Tokyo, Japan).

Quantitative real-time reverse transcription-PCR (RT-PCR). Total RNA was isolated by an RNeasy kit (Qiagen, CA) and reverse transcribed into cDNA by a SuperScript III kit (catalog no. 11752; Invitrogen, Carlsbad, CA). Quantitative PCRs were run in an ABI Prism 7700 Fast sequence detector system as previously described (26). The levels of mRNA were determined by using cataloged primers (Invitrogen) for human TNF-α (Hs00174128_m1), IL-1β (Hs01555410_m1), and 18S (Hs99999901_s1). Results were expressed as the relative fold difference.

Histology scoring. Epithelial cell damage in response to C. difficile toxins in human colonic explants was graded using a scoring system as previously published (27), and a score of 0 to 3 was assigned. Images from hematoxylin-eosin (H&E)-stained tissue sections were recorded at 100× magnification.

Statistical analyses. Quantitative results are expressed with error bars as mean ± standard error of the mean. Results were analyzed using the Prism professional statistics software program (GraphPad, San Diego, CA). Student’s t tests with Mann-Whitney posttests were used for intergroup comparisons.

RESULTS
MK3415 and MK6072 inhibit toxin A- and toxin-B induced TNF-α expression in human PBMCs. As shown in a recent report, toxin A induces expression of the proinflammatory cytokine...
TNF-α in human PBMCs (25). In the current study, toxin A at 0.1 and 1 μg/ml significantly increased TNF-α protein and mRNA expression in monocytes in a concentration-dependent manner (Fig. 1A and B). While pretreatment of PBMCs with control IgG 30 min prior to toxin exposure did not significantly alter toxin A-induced TNF-α expression compared to that for the control (no antibody), pretreatment with the anti-toxin A antibody MK3415 significantly reduced toxin A-induced TNF-α expression at the protein and mRNA levels (Fig. 1A and B). Pretreatment with MK3415 dose-dependently inhibited TNF-α protein and mRNA expression in PBMCs in the presence of 0.1 μg/ml toxin A (Fig. 1C and D).

Similarly, toxin B at 0.1 to 10 μg/ml induced TNF-α expression in human PBMCs (Fig. 2A and B). Toxin B at 1 μg/ml exerted the maximal response, while toxin B at 10 μg/ml stimulated modest TNF-α secretion (Fig. 2A). A similar lack of a dose-dependent response in macrophages in response to toxin B and toxin A was previously reported (26) and was probably related to cell death at high toxin doses. Pretreatment of PBMCs with control IgG did not significantly alter toxin B-induced TNF-α expression compared to that for the control, but pretreatment with the anti-toxin B antibody MK6072 significantly reduced toxin B-induced TNF-α mRNA and protein expression (Fig. 2A and B). MK6072 dose-dependently inhibited TNF-α protein and mRNA expression in PBMCs in the presence of 1 μg/ml toxin B (Fig. 2C and D).

**MK3415 and MK6072 inhibit toxin A- and toxin B-induced IL-1β expression in human monocytes.** Previous experiments indicated that toxins A and B induce IL-1β expression in macrophages (29, 30). Consistent with these findings, toxin A significantly induced IL-1β protein secretion by human PBMCs in the conditioned medium, with a maximal response at 0.1 μg/ml toxin B (Fig. 3A). A further increase of the toxin A concentration reduced IL-1β protein expression, although mRNA expression continued to increase (Fig. 3A and B). There was no significant difference in IL-1β protein and mRNA expression between the control IgG group and the control (blank) group. Pretreatment of PBMCs with the anti-toxin A antibody MK3415 significantly reduced toxin A-induced IL-1β protein and mRNA expression in these cells. MK3415 dose-dependently inhibited IL-1β protein and mRNA expression in PBMCs (Fig. 3B). Similarly, toxin B at 0.1 to 10 μg/ml induced IL-1β protein and mRNA expression in human PBMCs (Fig. 3C and D). MK3415 dose-dependently inhibited toxin B-induced IL-1β protein and mRNA expression in PBMCs (Fig. 3D).

**FIG 3** MK3415 attenuated toxin A-mediated IL-1β expression in PBMCs. (A and B) PBMCs were incubated with no antibody (blank), control IgG, or MK3415 (30 μg/ml) for 30 min, followed by incubation with toxin A at 0 to 1 μg/ml for 4 h. (C and D) PBMCs were incubated with control IgG or MK3415 (0.1 to 50 μg/ml) for 30 min, followed by incubation with toxin A at 0.1 μg/ml for 4 h. Conditioned media were collected for IL-1β ELISA. IL-1β mRNA expression was detected by real-time RT-PCR. All experiments are representative of 3 independent experiments.
mRNA expression in PBMCs in the presence of 0.1 μg/ml toxin A (Fig. 3C and D).

Toxin B at 1 μg/ml also induced maximal IL-1β protein and mRNA expression in PBMCs (Fig. 4A and B). Incubation of cells with anti-toxin B antibody MK6072 significantly inhibited toxin B-induced IL-1β protein and mRNA expression. MK6072 dose-dependently inhibited IL-1β protein and mRNA expression in PBMCs in the presence of 1 μg/ml toxin B (Fig. 4C and D).

**MK3415 and MK6072 inhibit toxin A- and toxin B-induced NF-κB p65 phosphorylation in human monocytes.** Toxin A mediates TNF-α expression via NF-κB activation (6, 7). We previously reported that toxin A can induce NF-κB p65 subunit phosphorylation in human PBMCs (25). Here, we found that toxin A induces p65 phosphorylation in the same cells using an ELISA approach, as described in Materials and Methods. Addition of MK3415 inhibited toxin A (0.1 to 1 μg/ml)-induced p65 phosphorylation in PBMCs (Fig. 5A). MK3415 dose-dependently reduced toxin A-induced p65 phosphorylation in PBMCs (Fig. 5B).

Exposure of these cells to different toxin B concentrations induced p65 phosphorylation, with higher levels of induction occurring at 1 μg/ml (Fig. 5C). Incubation with MK6072 reduced toxin B-induced p65 phosphorylation in PBMCs (Fig. 5C). MK6072 dose-dependently inhibited p65 phosphorylation in PBMCs in the presence of 1 μg/ml toxin B (Fig. 5D).

Toxin A and toxin B also activate the MAP kinase ERK pathway in mouse dendritic cells and colonocytes (8, 31). ERK activation is necessary for proinflammatory cytokine CXCL2 and chemokine IL-8 expression in response to toxin A and toxin B. We found that both toxin A and toxin B activated ERK phosphorylation in mouse Raw264.7 macrophages, while addition of control IgG did not alter this response (see Fig. S1A in the supplemental material). Moreover, addition of the anti-toxin A MAb MK3415 blocked toxin A-associated ERK phosphorylation in these cells (see Fig. S1A and B in the supplemental material). In addition, the anti-toxin B antibody MK6072 reduced toxin B-induced ERK phosphorylation (see Fig. S1C and D in the supplemental material).
MK3415 and MK6072 significantly inhibit toxin A- and toxin B-induced TNF-α and IL-1β mRNA expression in human colonic explants. Previous results indicated that toxins A and B can cause epithelial cell damage in human colonic explants (4). Here human colonic explants were divided into several pieces in Earle’s balanced salt solution buffer and incubated with toxin A or B and the respective human MAbs overnight. Toxin A and toxin B dose-dependently increased TNF-α mRNA in human colonic explants (Fig. 6A and B). Incubation with MK3415 significantly inhibited TNF-α mRNA expression induced by 0.01 to 1 µg/ml toxin A (Fig. 6A), while MK6072 inhibited toxin B (10 µg/ml)-induced TNF-α mRNA in human colonic explants (Fig. 6B).

Similarly, toxin A (0.1 to 1 µg/ml) and toxin B (10 µg/ml) induced IL-1β mRNA expression in human colonic explants (Fig. 6C and D). Incubation of the explants with MK3415 significantly inhibited the IL-1β mRNA expression induced by 0.1 to 1 µg/ml toxin A (Fig. 6C), while MK6072 inhibited toxin B (10 µg/ml)-induced IL-1β mRNA (Fig. 6D).

MK3415 and MK6072 attenuate toxin A- and toxin B-induced epithelial tissue damage in human colonic biopsy specimens. Consistent with our previous findings (4), toxins A and B elicited dramatic epithelial tissue damage in fresh human colonic explants (Fig. 7A and C). MK3415 significantly decreased toxin A-mediated epithelial cell damage (Fig. 7B), while MK6072 inhibited epithelial tissue damage in response to toxin B (Fig. 7D).

DISCUSSION

The Gram-positive anaerobe C. difficile mediates antibiotic-associated diarrhea and pseudomembranous colitis by releasing two large exotoxins, toxin A and toxin B (2, 32). Not only do these two toxins mediate intestinal inflammation and diarrhea in CDI pa-
MK3415 and MK6072 attenuated toxin A- and toxin B-mediated TNF-α and IL-1β mRNA expression in human colonic explants. (A) Fresh human colonic explants were incubated with control IgG or MK3415 (30 µg/ml) for 30 min, followed by incubation with toxin A at 0 to 1 µg/ml for 16 h. (B) Fresh human colonic explants were incubated with control IgG or MK6072 (30 µg/ml) for 30 min, followed by addition of toxin B at 0 to 10 µg/ml for 16 h. TNF-α mRNA expression was detected by real-time RT-PCR. (C) Fresh human colonic explants were incubated with control IgG or MK3415 (30 µg/ml) for 30 min, followed by incubation with toxin A at 0 to 1 µg/ml for 16 h. (D) Explants were incubated with control IgG or MK6072 (30 µg/ml) for 30 min, followed by addition of toxin B at 0 to 10 µg/ml for 16 h. IL-1β mRNA expression was detected by real-time RT-PCR. All experiments are representative of 5 patient samples.

The cellular and molecular mechanisms by which C. difficile toxins A and B mediate diarrhea and inflammation have been in good part elucidated by in vitro experiments and studies with relevant experimental models (2, 6). Studies also showed that pre-treatment with antibodies against these toxins is able to neutralize the responses in vitro and in vivo (20, 34–36). Such studies included experiments with human MAbs against these toxins (22). The beneficial effects of human anti-toxin A and B MAbs in vivo and in vitro (22) led us to hypothesize that these MAbs may prevent the proinflammatory responses to these toxins in human colon and human PBMCs through modulation of important signaling pathways that regulate the mucosal inflammation activated by these toxins. We report here that human MAbs exert their anti-inflammatory activity against toxins A and B in native human colon and human PBMCs by neutralizing the toxins and thereby inhibiting their effects on activation of host MAP kinase and NF-κB signaling pathways. Most of our experiments with PBMCs demonstrated that 30 to 50 µg/ml of anti-toxin A antibody MK3415 and anti-toxin B antibody MK6072 is sufficient to block >90% of toxin A- and B-mediated TNF-α and IL-1β expression, while their 50% effective dose is approximately 10 to 30 µg/ml (Fig. 1 to 4).

Our data confirmed our earlier findings that native human colon is sensitive to both C. difficile toxins (4) and for the first time showed that toxins A and B can stimulate TNF-α and IL-1β gene transcription in human colonic explants. Interestingly, our results also showed that human MAbs against these toxins significantly reduced toxin A- and toxin B-mediated TNF-α and IL-1β expression in native human colon and human PBMCs. Confirming this protective toxin A- and B-induced innate immune response, we
also showed that MK3415 and MK6072 also neutralized epithelial cell damage in human colonic explants in response to toxin A and B exposure, respectively.

We show here that anti-toxin A and B MAbs MK3415 and MK6072, respectively, diminish toxin-induced NF-κB p65 subunit and ERK phosphorylation in monocytes/macrophages. Both NF-κB and ERK phosphorylation are required to elicit secretion of proinflammatory cytokines in response to toxin A and B in vivo and in vitro (7, 8, 12, 13, 31). Therefore, these two toxin A- and toxin B-neutralizing antibodies possibly prevented the toxins from triggering upstream signaling pathways and the resulting cytokine production and tissue damage.

In summary, the human anti-toxin A and anti-toxin B antibodies MK3415 and MK6072, respectively, significantly reduce C. difficile toxin A- and toxin B-mediated proinflammatory cytokine expression and colonic epithelial tissue damage in native human
colitis. This report provides further support for the clinical application of these two antibodies in CDI.

ACKNOWLEDGMENTS

We thank Michelle Cheng, Ryan Ichikawa, Yuzu Kubota, Deanna Tran, and Irene Chang for assisting with our experiments. We also thank the Translational Pathology Core Laboratory, Department of Pathology, University of California at Los Angeles, for processing human colonic biopsy specimens for histologic assessment.

This work was supported by a research grant from Merck Pharmaceuticals (to C.P.). H.W.K. was supported by a Crohn’s and Colitis Foundation of America Career Development Award (no. 2691) and NIH grant K01 DK084256 grant. S.H. was supported by a Crohn’s and Colitis Foundation of America student research fellowship (no. 3831). Support was also provided by the Blinder Research Foundation for Crohn’s Disease (to C.P.), the Eli and Edythe Broad Chair (to C.P.), and United States Public Health Service grant DK046763 (to D.Q.S.).

C.P. is supported by NIH grant ROI AI095256, and X.C. is supported by a Career Development Award from the Crohn’s and Colitis Foundation of America.

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