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Phosphatidylcholine and the intestinal mucus layer: in vitro efficacy against *Clostridium difficile*-associated polymorphonuclear neutrophil activation

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Abstract

BACKGROUND: Phosphatidylcholine (PC), an important component of intestinal mucus, protects against *Clostridium difficile* toxin-induced intestinal barrier injury in vitro. Polymorphonuclear neutrophil (PMN) activation may contribute to intestinal injury and systemic toxicity in patients with *C. difficile*-associated disease. We therefore hypothesized that the intestinal barrier function against *C. difficile* toxin by exogenous PC would ameliorate PMN activation.

METHODS: Intestinal epithelial cell (IEC) monolayers were cocultured with *C. difficile* toxin A and/or exogenous PC. Naïve PMNs were cocultured with IEC culture supernatants and PMN activation, and chemotactic potential determined.

RESULTS: PC treatment of IEC abrogated the enhanced PMN activation and chemotactic potential following toxin A exposure ($P < .001$).

CONCLUSIONS: Exogenous PC ameliorated PMN activation from IECs exposed to *C. difficile* toxin. Administration of exogenous PC may be a useful adjunctive treatment in severely ill or immunocompromised patients with *C. difficile*-associated disease.

Clostridium difficile-associated disease (CDAD) has increased in incidence and severity especially since 2000. The increase in disease severity may in part relate to the emergence of epidemic strains with enhanced virulence properties.[1] However, host proinflammatory and humoral immune responses are also important in limiting disease severity and risk of mortality with CDAD.[2]

It is now recognized that new therapies are needed for the treatment of CDAD, especially in patients with severe or recurrent disease. In this regard, the colonic mucus layer has been demonstrated to be an important component of the innate barrier that prevents colonic bacteria from invading the mucosa and causing inflammation. In a previous study, we demonstrated that the mucus layer acts in a synergistic fashion with secretory IgA (the main antibody in intestinal mucosal secretions) to protect against *C. difficile* toxin-induced intestinal injury.[3]

Phosphatidylcholine (PC) is an important component of the mucus layer and it has intrinsic anti-inflammatory properties.[4] Decreased PC content in the intestinal mucus

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has been demonstrated in patients with chronic ulcerative colitis (CUC).[5] This finding lead to the clinically effective use of a delayed release formulation of PC in recently published trials in CUC.[5] In a prior in vitro study, we demonstrate that exogenous PC supplementation protects against intestinal barrier injury after *C. difficile* toxin exposure.[6] This was evident even in experiments with an intact mucus layer.

Intestinal epithelial cell (IEC) neutrophil interactions are important in mucosal inflammation.[7] In our prior study, there was a 3-fold to 4-fold decrease in the proinflammatory cytokines tumor necrosis factor alpha and interleukin 6 (IL-6) with IECs supplemented with PC and exposed to *C. difficile* toxin A.[6] This may be important as proinflammatory signaling from the gut is a trigger for neutrophil trafficking to the gut, which may increase intestinal barrier injury. Modulation of neutrophil–epithelial cell interaction may be a novel approach in the treatment of severe forms of CDAD. We therefore hypothesized that exogenous PC supplementation would ameliorate polymorphonuclear neutrophil (PMN) inflammatory responses and chemotactic potential. This was studied in an in vitro model.

Patients and Methods

Intestinal epithelial cells

HT29 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% CO_2 at 37°C. Cells (5×10^5) were seeded on the apical surface of a polycarbonate membrane (3.0-μm pore size) (Transwell; Corning Costar Core, Cambridge, MA) in a 2-chamber cell culture system and allowed to form polarized monolayers. Monolayer integrity was monitored by serial measurement of the transepithelial electrical resistance with a Millicell electrical resistance meter (Millipore Corp., Bedford, MA).

HT29 methotrexate cells

The HT29 MTX cell line was isolated from human HT29 colon carcinoma cells through growth adaptation to methotrexate (MTX) as described by Olson et al.[6] Briefly, exposure of HT29 cells to 10^{-7} M MTX led to differentiation into a homogeneous monolayer of polarized goblet cells, which secrete mucins of gastric immunoreactivity. After stabilization of the growth curve, cells adapted to 10^{-7} M MTX after passage 8 were reverted back to drug-free medium and subsequently cultured in the absence of MTX. The growth curve remained unchanged as compared with the MTX-treated cells. In our previous study, exogenous PC protected the intestinal barrier against *C. difficile* toxin A in both mucus-producing and non–mucus-producing HT29 clones. Thus, we used both HT29 clones in this study to extend these findings to PMN responses in this model.

Polymorphonuclear neutrophil isolation

Venous whole blood was collected from random healthy donors in vacuum tubes containing ethyenediamine tetra-acetic acid. PMNs were isolated by first incubating the whole blood with 6% dextran for 45 minutes at 4°C. The leukocyte-rich supernatant was aspirated and layered on top of Histopaque 1077 (Sigma, St. Louis, MO) and centrifuged at 1,300 rpm (400*g*) for 30 minutes at 4°C. A 25-second red blood cell lysis was performed on the pellet and isotonicity restored by adding 2 mL of 3.4% NaCl. The cells were then diluted in phosphate buffered saline (PBS) and centrifuged for 10 minutes at 1,300 rpm at 4°C. The pellet was washed and gently resuspended in PBS at a concentration of 1×10^6 cells/mL and used immediately.

Experimental design

Confluent HT29 and HT29-MTX IECs were first established in a 2-chamber cell culture system. *Clostridium difficile* toxin A (50 μg/mL) was cocultured with the IEC for 6 hours. In a subset of experiments, phosphatidylcholine (PC) (100 μM) was cocultured with the IECs for 1 hour before exposure to *C. difficile* toxin A. Basal chamber supernatants from HT29 and HT29-MTX monolayers exposed to toxin A and PC as described above were collected and cocultured with naïve PMNs freshly isolated from healthy volunteers, and PMN activation was indexed by CD11b expression (MFI), superoxide anion generation (O_2^-) after addition of N-formylmethionyl-leucyl-phenylalanine (fMLP) (activation), and percent elastase release. Chemotaxis of PMNs was analyzed following incubation of IEC culture supernatants (with or without *C. difficile* and PC) with PMNs in a chemotaxis system.

CD11b adhesion molecule quantification

Mean receptor density on the PMN plasma membrane was quantified by flow cytometry with a fluorescent (phycoerthrin)-labeled monoclonal antibody directed against the CD11b receptor (BD Biosciences Pharmingen, San Diego, CA). Briefly, PMNs were cocultured with HT29 or HT29-MTX supernatants for 60 minutes at 37°C and then isolated by centrifugation and subsequently incubated with a 1:100 dilution of the anti-CD11b antibody for 30 minutes at 4°C in the dark. Control samples were stained separately with a mouse isotype control antibody to assess background antibody binding. The PMNs were then washed with cold PBS containing 0.5 mM glucose and .1% gelatin and fixed with 1% paraformaldehyde in PBS. The data are reported as mean fluorescence intensity, reflecting the mean CD11b receptor density on the PMN cell surface.