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MAIN TOPIC

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The effect of phospholipids and mucin on bacterial internalization in an enterocyte-cell culture model

Abstract A primary component of the intestinal mucous layer that functions as a barrier to luminal bacteria is mucin, a high-molecular-weight glucoprotein. In addition, the mucous layer also contains other important elements such as phospholipids (PLs), which may effect bacterial translocation (BTL). It has been reported that mucin inhibits *Escherichia coli* translocation; however, the effect of PLs on intestinal permeability is still controversial. We have recently reported that the concentration of mucous PLs is higher in neonatal as compared to adult rabbits. The functional significance of these biochemical differences on BTL remains to be determined. The aim of this study was to evaluate the effect of PL and mucin composition on BTL in a human enterocyte-cell culture model. Human enterocyte Caco-2 cells were seeded in 24-well tissue-culture plates and grown for 14 days to confluence. The monolayers were pretreated with phosphate buffered saline as control, 10 mg/ml or 20 mg/ml mucin, 0.5 mM or 1.0 mM PL mixture based on neonatal (NPL) and adult (APL) composition, and 10 mg/ml mucin with 0.5 mM either APL or NPL mixtures 30 min before a 120-min incubation period at 37 °C with 10^8 colony forming units (CFU) of *E. coli* C25. Non-internalized bacteria were killed by the addition of gentamicin. Internalized bacteria were quantified by counting CFU from enterocyte-cell lysates grown on MacConkey's agar. Mucin inhibited bacterial internalization, while both compositions of PLs promoted it. Mucin added to the PL solution also diminished the stimulatory effect of PLs on bacterial internalization. These results indicate that the increased concentration of PLs found in the intestinal mucous layer of neonates, and/or the alteration in the balance between PLs and mucin, may play a role in the increased BTL seen in neonates.

Key words Bacterial translocation · Mucous layer · Phospholipids · Mucin · Caco-2 cell

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Introduction

Some investigators have suggested that bacterial translocation (BTL) may be a major cause of neonatal sepsis [27]. We have previously reported that neonatal rabbits have a higher incidence of spontaneous BTL than adult rabbits [26, 28]. However, the cause of this increased incidence in neonatal rabbits remains unclear. Multiple factors may affect the neonate's host-gut defense mechanisms: an immature immune system, epithelial cells, gastric acidity, enzyme availability, intestinal motility, and the composition of the mucous gel layer (MGL) [1, 10, 25, 27].

The MGL, which overlies the mucosal surface, is composed of water and electrolytes, various serum and cellular macromolecules including secreted immunoglobulins [2], cell debris, phospholipids (PL), and high-molecular-weight glycoproteins known as mucins [17]. Differences in these components between adult and neonatal animals may explain the mechanism of the higher incidence of BTL observed in infants. The mucin composition in adult animals has been shown to be different from that found in neonatal animals [23]. Moreover, we have recently reported that the concentration and composition of mucous PLs in neonatal rabbits is different from that in adult rabbits [18]. Several investigators have reported that mucin inhibits *Escherichia coli* BTL [6, 15]; however, the effect of PL on intestinal permeability is still controversial [3, 7, 25, 31]. Therefore, the functional significance of mucin and PL biochemical compositions on BTL remains to be determined. The aim of this study was to evaluate the effects of PL and mucin on bacterial internalization (BINT),

which is thought to be the initial step of BTL, in an enterocyte-cell culture model.

Materials and methods

Escherichia coli C 25, a nonpathogenic, streptomycin-resistant strain originally isolated from human gut flora, was used for these studies (kindly provided by Dr. Henri R. Ford, Pittsburgh, PA). The *E. coli* C 25 were grown overnight in brain-heart infusion medium (BBL, Cockeysville, MD), washed two times with phosphate buffered saline (PBS), and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) at a concentration of 10^9 colony forming units (CFU)/ml. The initial concentration of bacteria was determined spectrophotometrically at a wavelength of 650 nm and the number of bacteria was verified by pour-plate assay using MacConkey's agar (Difco, Detroit, MI) with serial dilution.

Human colonic carcinoma (Caco-2) cells were obtained from the American Type Culture Collection No. HTB 37 (Manassas, VA). Caco-2 cells are transformed human colon carcinoma cells that display many features of differentiated small-intestinal enterocytes [9, 20]. They spontaneously form polarized monolayers and tight junctions and the apical surface of the cells has well-developed microvilli, which contain disaccharidases and peptidases typical of normal small-intestinal villous cells [9, 20]. Moreover, the Caco-2 cell line has been used to study enterocyte interactions with bacteria such as *E. coli* [16, 19, 32]. Cell passages 24–37 were grown in DMEM supplemented with 10% fetal bovine serum (Gibco), 1% non-essential amino acid solution (Gibco), 1% sodium pyruvate (Gibco), penicillin G (100 IU/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere at 37 °C. They were harvested after reaching 60%–70% confluence following trypsinization with trypsin-EDTA (Gibco), washed, and resuspended in DMEM. The cells were seeded at a density of 5×10^4 per well (2 cm^2) onto collagen-coated 24-well tissue-culture plates (Corning, Corning, NY). Collagen coating of the plates was accomplished by incubating in 30 μl 0.25 mg/ml rat-tail Type I collagen (Sigma, St. Louis). The cells were grown for 14 days to allow them to reach to reach confluence and fully differentiate. Media were changed every 2nd day.

Intracellular bacteria were measured according to previously published methodology [11, 31], with minor modifications. Briefly, prior to addition of bacteria, the Caco-2 monolayers were washed three times with DMEM without fetal bovine serum or antibiotic supplements and pre-incubated for 30 min in fresh DMEM containing one of the following: PBS as control, mucin (type III, Sigma) 10 mg/ml or 20 mg/ml PL mixture based on neonatal composition (NPL; 0.5 mM or 1 mM), PL mixture based on adult composition (APL; 0.5 mM or 1 mM), and mucin with both types of PL mixture (10 mg/ml mucin with 0.5 mM NPL or APL). Mucin was dissolved in PBS to form a solution. The neonatal PL mixture consisted of 40% phosphatidylcholine (PC), 40% phosphatidylethanolamine (PE), 10% sphingomyelin (SM), and 10% phosphatidylserine (PS). The adult PL mixture consisted of 20% PC, 20% lyso-PC, 20% PE, 20% lyso-PE, 10% SM, and 10% PS. The composition of neonatal versus adult PL was based on our previous study in rabbits [18].

The neonatal and adult PL mixtures were added as liposomes in PBS using an ultrasonic cell disrupter (Kontes). 100 μl containing 10^8 CFU bacteria were added to each well containing Caco-2 monolayers and pre-incubated for 30 min in the appropriate solution. After a 120-min incubation at 37 °C in 5% CO_2, the monolayers were washed five times with PBS followed by the addition of DMEM containing 100 μg/ml gentamicin to kill any remaining viable extracellular bacteria. It has been reported that *E. coli* can survive within Caco-2 cells [32] and that gentamicin (100 μg/ml) is not transported by Caco-2 cells, and thus should not affect the viability of intracellular bacteria [21]. After 120 min, the monolayers were washed five times with PBS and lysed for 10 min with 0.5% Triton X-100 (Sigma). The number of viable bacteria in the lysates was quantitated by pour-plate assay using MacConkey's agar.

The number of *E. coli* was expressed as log CFU per cm^2 monolayer. Statistical analysis was performed by Kruskal-Wallis test with post-hoc Wilcoxon rank-sum test. Data were expressed as mean ± SEM, and statistical significance was defined as $P < 0.05$.

Results

Bacterial internalization by Caco-2 enterocytes was investigated in control ($n = 42$) and mucin ($n = 23$)-treated monolayers. Internalization was significantly inhibited ($P < 0.05$) by 10 mg/ml ($n = 14$) and 20 mg/ml ($n = 9$) mucin solution compared to control monolayers (Fig. 1). There was no significant difference in the degree of inhibition as measured by BINT between 10 mg/ml and 20 mg/ml mucin. This inhibitory effect of mucin on BINT confirms the previous work of Cruz et al., who demonstrated decreased BTL across the Caco-2 monolayer by 8 mg/ml purified mucin [6].

The effect of PL on BINT in Caco-2 enterocytes was also investigated using physiologic mixtures of PL. Internalized bacteria were measured at two different PL concentrations: 0.5 ($n = 16$) and 1 mM ($n = 8$). BINT was significantly increased in monolayers treated with both neonatal and adult PL ($P < 0.05$) (Fig. 2). There was no significant difference in BINT between NPL and APL at either concentration.

The relationship between mucin and both NPL and APL on BINT by Caco-2 enterocytes was investigated. BINT following treatment with 0.5 mM NPL ($n = 14$) or 0.5 mM APL ($n = 8$) plus 10 mg/ml mucin is presented in Fig. 3. The number of internalized bacteria with treatment by either neonatal or adult PL alone was significantly higher than that of controls. In contrast, the number of internalized bacteria treated with mucin plus either NPL or APL was significantly lower than both

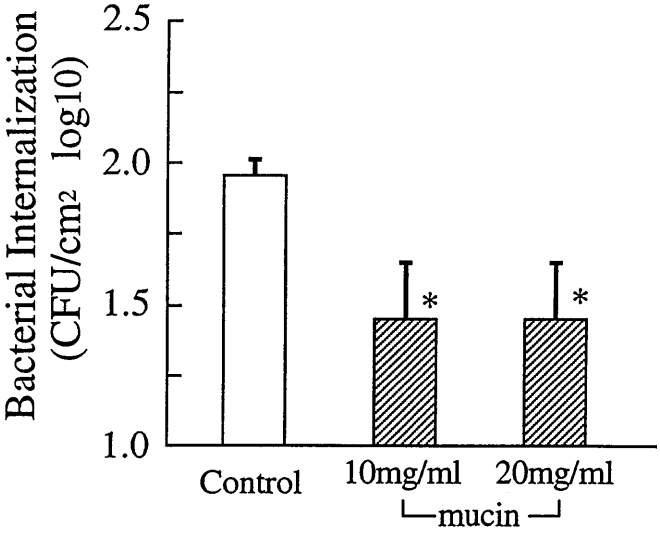

Fig. 1 Effect of mucin concentration on number (mean ± SEM) of internalized *E. coli* C25 [expressed as log(CFU/cm^2)] within Caco-2 enterocyte monolayers (*CFU* colony-forming units)
*$P < 0.05$ vs. control

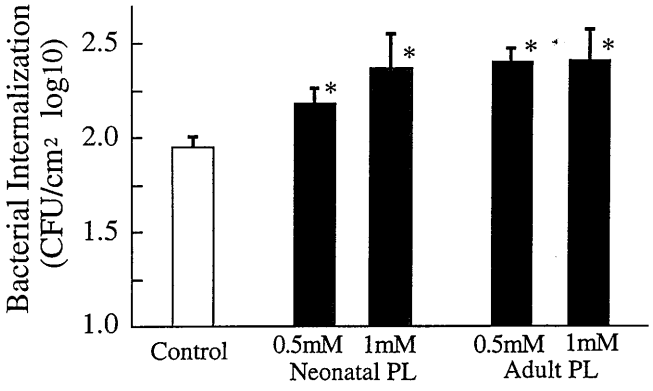

Fig. 2 Effect of neonatal and adult phospholipid (PL) concentrations on number (mean ± SEM) of internalized *E. coli* C25 [expressed as log(CFU/cm^2)] within Caco-2 enterocyte monolayers (*CFU* colony-forming units)
*$P < 0.05$ vs. control

controls and NPL or APL alone. These results suggest that the stimulatory effects of neonatal and adult PL on BINT were overshawdowed by the inhibitory effects of mucin.

Discussion

One of the major causes of neonatal mortality is sepsis of unknown etiology [8]. It has been suggested that BTL may be a major cause of neonatal sepsis, especially when no primary source of infection is identified [27]. We have previously demonstrated that neonatal rabbits have a higher incidence of spontaneous BTL than adult rabbits [26, 28]. However, the reasons behind the higher incidence in these neonates remain unclear.

Many factors may affect BTL in neonates, including colonization of the gut, immaturity of the immune system, immaturity of epithelial cells, gastric acidity, gastric enzymes, intestinal motility, and the mucous gel layer (MGL) overlying the mucosal surface [10, 27]. Of these factors, the MGL is also known to be an important component of the host-gut defense barrier [1, 2, 25]. It is composed of water and electrolytes, various serum and cellular macromolecules including secreted immunoglobulins [2], cell debris, PLs, and high-molecular-weight glycoproteins known as mucins [17]. The differences in these components may explain the higher incidence of BTL in neonates. Our studies have focused on the differences in concentration and composition of PLs and mucin that exist within the mucous layer. The composition of mucin in adult animals has been shown to be different from that of neonatal animals [23], and we have recently reported that the concentration and composition of mucous PL in neonatal rabbits is different from that in adult rabbits [18]. However, the functional significance of these biochemical differences on BTL remains to be determined.

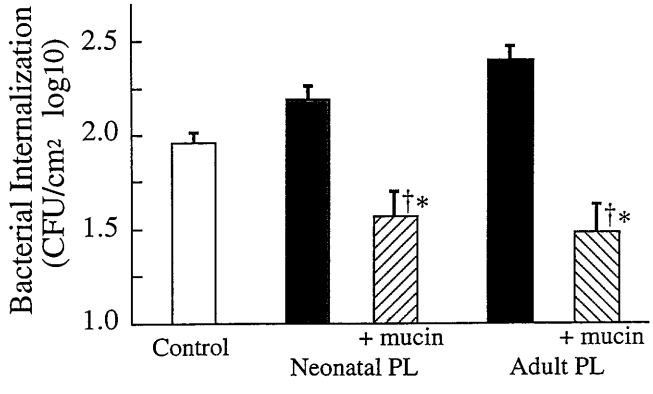

Fig. 3 Effect of mucin and neonatal or adult phospholipid (PL) on number (mean ± SEM) of internalized *E. coli* C25 [expressed as log(CFU/cm^2)] within Caco-2 enterocyte monolayers (*CFU* colony-forming units)
*$P < 0.05$ vs. neonatal or adult PL without mucin
†$P < 0.05$ vs. control

It has been reported that intestinal mucin binds to *E. coli* and inhibits its enterocyte adherence. Binding was noted to be dependent on the intestinal sites that secrete the mucin as well as the age of the animal [13, 29]. The mechanism of this binding has been suggested as follows: carbohydrate moieties on the mucin molecule competitively bind and trap pathogens by either mimicking epithelial cell-membrane glycoproteins, which are recognized by a pathogen's adherence lectin [5], or by binding to other membrane components such as type 1 pili expressed by *E. coli* [22]. Experimentally, it has been reported that mucin inhibits *E. coli* BTL in a rat mucosal model [15] and a cultured enterocyte model [6]. In the latter experiment, 8 mg/ml purified mucin inhibited the rate of *E. coli* translocation across the Caco-2 monolayer, but 1 mg/ml and 5 mg/ml did not [6]. In our study, it was shown that 10 mg/ml mucin solution inhibits *E. coli* internalization by Caco-2 cells, which is consistent with the previous reports.

One of the other important components of the mucous layer is PL. It has been reported that PL surfactant contributes to the mucosal barrier function of the gastric MGL [4, 12]. In addition, protective effects of bile, which includes several kinds of PL, against BINT have been reported in a cultured human enterocyte model [31]. Also, the effect of PL administered orally on the inhibition of BTL has been described in a rat model [7]. In contrast, it has been shown that several kinds of surfactants, including bile acids, enhanced intestinal epithelial permeability to mannitol and polyethylene glycol in monolayers of Caco-2 cells [3]. Additionally, high concentrations of lysophosphatidylcholine, one of the typical mucus PLs, increased gut permeability to macromolecules in rat epithelium [25]. Thus, there are conflicting data regarding the effect of PL on intestinal permeability.

The controversial reports in the literature and our own previous experimental data on the concentration and composition of mucosal PL stimulated us to carry out the present studies to test the effect of PL on BTL in a different model and to test the combined effects of

mucin and PL. In our experiments, both the neonatal and adult mixtures of PL, which were given as liposomes suspended in PBS, promoted BINT. Bengmark and Jeppsson [4] reported that PLs are present in the gastrointestinal tract: (1) at the surface of the mucous layer; (2) at the surface of the mucous cells as liposome-like aggregates within the mucus; and (3) at the surface of the mucous cells. Lichtenberger [12] reported that the MGL contains PL as lamellar, vesicular, and filamentous structures. From this point of view, the PL liposome made in PBS may not physiologically imitate the indigenous PL found in vivo.

In order to determine the effect of mucin on the stimulatory effect of PL, BINT was investigated with exposure to PL and mucin mixed together in solution. Our results suggest that the stimulatory effect of PL on BINT was diminished by the addition of mucin. Considering our previous report, which showed that the concentration of mucous PL is higher in neonatal rabbits than in adult rabbits [18], these results suggest that an alteration in the balance between PL and mucin may play a role in the increased BTL seen in neonates. Alternately, PL has been known to affect the physical properties of mucus by decreasing its viscosity and elasticity [14], and increasing the solubility of mucus, which may affect BTL [24]. More detailed studies may be necessary to understand how the interaction between PL and mucin contributes to the high incidence of spontaneous BTL seen in the newborn.

In summary, our results indicate that the increased concentration of PLs found in the intestinal mucous layer of neonates and/or the alteration in the balance between PLs and mucin may play a role in the increased BTL seen in neonates.

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