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MAIN TOPIC

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The effect of phospholipase A_2 on bacterial translocation in a cell culture model

Abstract The activity of *phospholipase* (PL)A_2 is elevated in the intestinal epithelia of patients with inflammatory bowel disease (IBD). Recently, we reported that lysophosphatidylcholine (L-PC), the PLA_2 hydrolysis product of phosphatidylcholine (PC), stimulates bacterial translocation (BT) in an enterocyte cell-culture model. These two observations stimulated us to examine the effects of extracellular PLA_2 on intestinal epithelial permeability. Human Caco-2 enterocytes were grown to confluence on porous filters in the apical chamber of a two-chamber cell-culture system. Monolayer integrity and tight-junction permeability were measured by dextran blue (DB) permeability and transepithelial electric resistance (TEER). Monolayers were treated with PC, L-PC, or PLA_2 with and without PC. The magnitude of BT was determined 2 h after treatment by adding *Escherichia coli* to the apical chamber followed by quantitatively culturing basal chamber samples. Thin-layer chromatography (TLC) was utilized to verify PLA_2 hydrolysis of PC to L-PC. Statistical analysis was performed by one-way analysis of variance. The magnitude of BT across monolayers pretreated with PLA_2 + PC significantly increased compared to either PC or PLA_2 (6.83 ± 0.069, 2.41 ± 0.46, and 3.06 ± 1.14 log10 colony forming units/ml, respectively, $P < 0.05$). Absence of DB-permeability in any group confirmed monolayer integrity. TLC of PL samples harvested from the apical monolayer surface confirmed PC hydrolysis. PLA_2 mediates hydrolysis of PC to L-PC when both are applied to the apical surface of cultured enterocyte monolayers, resulting in increased BT and increased TEER with no damage to monolayer integrity. These observations may have implications in the pathogenesis and treatment strategies for IBD.

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Introduction

Traditionally viewed as an organ of nutritional absorption, the gut also has complex defense mechanisms to prevent bacteria from entering the systemic circulation. The mucous layer is believed to protect the intestinal epithelium from potentially pathogenic bacteria. Alterations in the mucous layer may promote bacterial translocation (BT) under pathologic conditions such as gut immaturity, ischemia-reperfusion, starvation, bacterial stasis and overgrowth, and inflammatory bowel disease (IBD) [11]. We have previously reported that intestinal mucous phospholipid concentrations and content are altered in neonatal rabbits compared to adult rabbits [9]. We have also recently reported that lyso-phosphatidylcholine (L-PC), the hydrolysis product of phospatidylcholine (PC), the most prominent phospholipid in the mucous layer, promotes BT in an enterocyte-cell culture model.

Phospholipase A_2 (PLA_2) comprises a family of lipolytic enzymes that catalyze the hydrolysis of the fatty acyl ester bond at the sn-2 position of glycero-3-phospholipids such as PC to produce free fatty acids and lysophospholipids such as L-PC. PLA_2 within the lumen of the intestine is derived from the pancreas and from mucosal Paneth-cell secretion (secretory PLA_2). Pancreatic PLA_2 functions in dietary and biliary phospholipid digestion whereas secretory PLA_2 functions within or on the exofacial surface of intestinal mucosal epithelia. It is clear that secretory PLA_2 plays an important role in a wide variety of inflammatory diseases, including septic shock, acute lung injury, inflammatory arthritides, and multisystem organ failure. Several human studies have documented that the activity of secretory PLA is elevated in the intestinal mucosa and serum of patients with both Crohn's disease and ulcerative colitis [6–8, 10].

The pathophysiologic role of PLA_2 in intestinal inflammation is not fully understood, however, most evidence suggests that the enzyme acts on cellular membrane phospholipids, generating pro-inflammatory molecules such as arachidonic acid and its metabolites (prostaglandins and leukotrienes) and lysophospholipids and their metabolites (platelet activating factor). Limited information is available regarding the possible role that luminal PLA_2 might play in intestinal disease by hydrolyzing extracellular or mucous phospholipids. The aim of this study utilizing an enterocyte monolayer model, was to examine whether PLA_2 can hydrolyze PC to the biologically active L-PC and then exert a biologic effect on parameters that accompany many intestinal inflammatory conditions such as intestinal epithelial permeability and BT.

Materials and methods

Materials included Caco-2 cells (American Type Culture Collection No. HTB 37, Manassas, VA), *Escherichia coli* C 25 (provided by Dr. Henri R. Ford, Pittsburgh, PA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, non-essential amino acids solution, sodium pyruvate, penicillin G, streptomycin, and trypsin-EDTA (Gibco, Grand Island, NY), a two-chamber cell-culture system (CCS), chloroform, methanol, $CuSO_4$ pentahydrate, concentrated H_3PO_4, and MacConkey's agar (Fischer, Pittsburgh, PA), rat tail type I collagen, dextran blue, PC, and L-PC (Sigma, St Louis, MO), PLA_2 (*Naja naja* cobra venom, Sigma), brain-heart infusion medium (BBL, Cockeysville, MD), and precoated silica gel plates (TLC plates, Silica gel 60, EM Science, Gibbstown, NJ).

Caco-2 cells are transformed human colon carcinoma cells that display many features of differentiated small-intestinal enterocytes. They spontaneously form polarized monolayers with tight junctions, and the apical surface of the cells has well-developed microvilli that contain disaccharidases and peptidases typical of normal small-intestinal villous cells. The Caco-2 cell line has been used extensively to study enterocyte interactions with bacteria such as *E. coli*.

Cell passages 26–37 were grown in DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids solution, 1% sodium pyruvate, penicillin G (100 IU/ml) and streptomycin (100 g/ml) in a 5% CO_2 atmosphere at 37 °C. After reaching 60%–70% confluence, cells were harvested by trypsinization with trypsin-EDTA, washed, and resuspended in DMEM, and then seeded at a density of 1×10^4 cells per well (0.33 cm^2) onto collagen-coated porous filters in the apical chamber of a two-chamber CCS. Collagen coating of the plates was accomplished by incubation in 30 l 1.0 mg/ml rat-tail type I collagen. The cells were then grown for 14 days in DMEM to allow them to reach confluence and fully differentiate. Media were changed every 2nd day.

PC and L-PC liposomes were prepared by dissolving each PL in chloroform:methanol (2:1), drying under a stream of dry nitrogen, resuspending in PBS, and subjecting them to sonication (Branson Sonifier 450; Danbury, CT).

Transepithelial electrical resistance (TEER), a measure of monolayer integrity and tight-junction permeability, was measured before and 2 h after adding the following: PLA_2 alone (0.2, 1.0, 5.0 IU/ml) in phosphate buffered saline (PBS), PC alone (2 mM), L-PC alone (2 mM), or PLA_2 (0.2, 1.0, 2.5, 5.0 IU/ml) + PC (2 mM) using an epithelial voltohmmeter (EVOM; World Precision Instruments, Sarasota, FL) and expressed as the ratio of post- to pre-TEER. TEER values obtained in the absence of cells were considered as background, and resistances were calculated in Ω/cm^2.

Translocated bacteria were measured according to previously published methodology [5, 13] with minor modifications. Briefly, *E. coli* C 25, a nonpathogenic, streptomycin-resistant strain originally isolated from human gut flora, were grown overnight in brain-heart infusion medium, washed three times with PBS, and resuspended in PBS at a concentration of 1×10^7 colony forming units (CFU) per milliliter. The initial concentration of bacteria was determined spectrophotometrically at a wavelength of 650 nm.

Prior to addition of bacteria, the Caco-2 monolayers were washed three times with DMEM without fetal bovine serum or antibiotic supplements. PLA_2 alone, PC alone, L-PC alone, or PLA_2 + PC were added onto the Caco-2 cell monolayers. After 30 min stabilization, *E. coli* C 25 were inoculated by adding 100 µl DMEM containing 1×10^6 CFU bacteria into the apical media. Following an additional 120 min incubation at 37 °C in 5% CO_2, samples from the basal chambers were taken and the number of bacterial CFU was determined by the pour-plate assay using MacConkey's agar. The magnitude of BT was expressed as $\log_{10}$[CFU/ml].

Dextran Blue (DB) (2,000,000 molecular weight) was used to verify the integrity of Caco-2 cell monlayers. PBS (600 µl) was added to the basal chambers, followed by the addition of the PLA_2 and PL liposomes. A 50-µl aliquot of DB solution dissolved in PBS (20 mg/ml) was added to the apical chamber. Following 120 min of incubation, a 500-µl sample from the basal chamber was taken and DB permeability across the Caco-2 monolayer was quantified by spectrophotometry (620 nm) and comparison made to a standard curve.

Thin-layer chromatography (TLC) was utilized to measure PLA_2 hydrolysis of PC to L-PC. Phospholipids were extracted using a modification of the method described by Bligh and Dyer [2]. After 120 min of incubation of PL liposomes and/or PLA_2 in the apical chamber overlying the Caco-2 monolayer, 100-µl samples from the chamber were transferred to glass tubes with Teflon screw caps; 3 ml chloroform:methanol (2:1) and 700 µl PBS were added and vortexed for 30 s. The upper layer of each tube was discarded and 1.0 ml methanol and 0.8 ml 0.9% NaCl were added and vortexed for an additional 30 s. After the upper layer of each tube was discarded by suction, the extracted lipid in the lower layer was evaporated to dryness under a stream of nitrogen and resuspended in 0.5 ml chloroform:methanol (2:1) and subjected to TLC; 10 µl of each sample was spotted on pre-coated silica gel plates. Spot separation was performed on 10×20-cm glass plates in solvent-saturated chambers. The solvent system consisted of chloroform-methanol-7 N ammonia (60:35:5). After 40 min, the plates were dried, sprayed with 8% (w/v) $CuSO_4$ pentahydrate in water/methanol/concentrated H_3PO_4 (60:32:8), and charred for 15 min at 150 °C. Each spot was recorded with a digital camera (DC 120 ZOOM, Kodak).

Statistical analysis was performed by one-way analysis of variance. Data were expressed as mean ± standard error of the mean, with statistical significance defined as $P < 0.05$.

Results

Effect on transepithelial electrical resistance

As has previously been shown, the TEER ratio (post-TEER/pre-TEER) of Caco-2 monolayers pretreated with L-PC (2 mM) significantly decreased compared to those treated with PC (2 mM) and PBS (Fig. 1). However, PC (2 mM), the most prominent PL found in mucus, had no effect on TEER compared to PBS controls. At the lowest concentration tested (0.2 IU/ml), PLA_2 alone had a small but significant effect to increase TEER compared to PBS. PLA_2 at higher concentrations

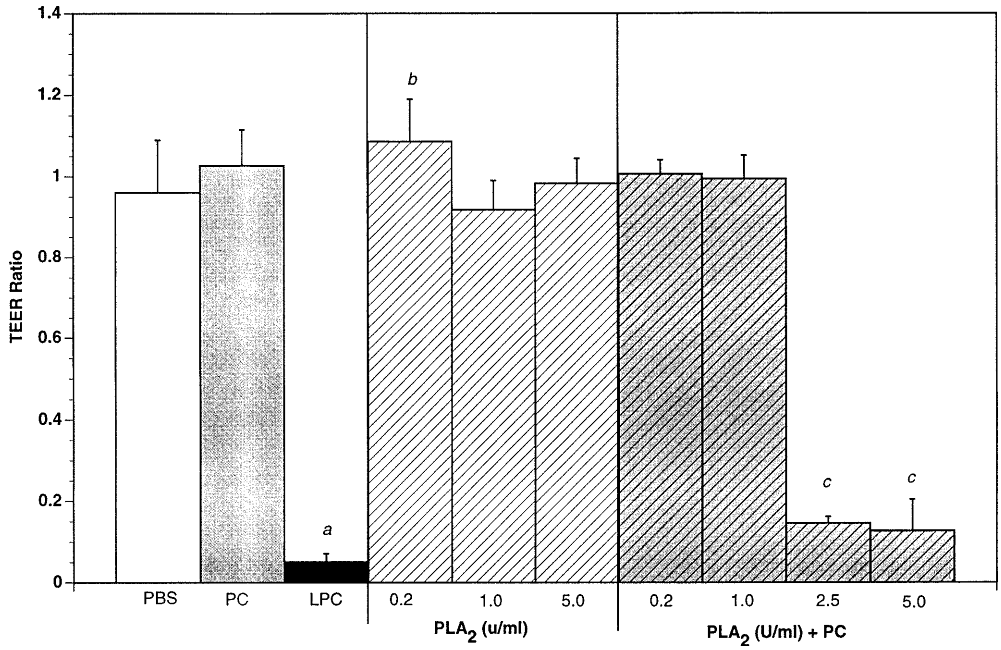

Fig. 1 Effects of phosphatidylcholine (PC), lysophosphatidyl choline (L-PC), phospholipase A_2 (PLA_2) and PLA_2 + PC on transepithelial electrical resistance (TEER) across Caco-2 monolayers measured before and 2 h after addition of PLs and/or PLA_2. (Data expressed as mean ± SD, *PBS* phosphate-buffered saline) *a* $P < 0.05$ vs PBS and PC *b* $P > 0.05$ vs PBS, *c* $P < 0.05$ vs PC; PLA_2 alone and 0.2, 1.0 PLA_2 + PC

(1.0 and 5.0 IU/ml) had no significant effect on TEER compared to controls. When PC (2 mM) was added with PLA_2 at various concentrations, a significant decrease in TEER was found at PLA_2 concentrations of 2.5 and 5.0 IU/ml.

Effect on BT

PC alone (2 mM) appeared to have a small inhibitory effect on BT across Caco-2 monolayers compared to PBS, however, this change did not reach statistical significance ($P = 0.053$) (Fig 2). L-PC (2 mM), on the other hand, significantly stimulated BT across Caco-2 monlayers compared to PC alone (2 mM) and PBS. Interestingly PLA_2 alone at the low concentration (0.2 IU/ml) significantly inhibited BT compared to PBS controls. Higher concentrations of PLA_2 had no significant effect on BT compared to controls. When PC (2 mM) was added with PLA_2 across a range of concentrations, BT increased in a dose-dependent manner, reaching statistical significance at 5.0 IU/ml PLA_2. The magnitude of BT was similar between L-PC alone and PLA_2 (5.0 IU/ml) plus PC.

DB permeability

To test whether the effect of L-PC and PLA_2 + PC on TEER and BT were secondary to enterocyte monolayer disruption, permeability to the macromolecular marker DB was determined. In order to establish a basal rate of

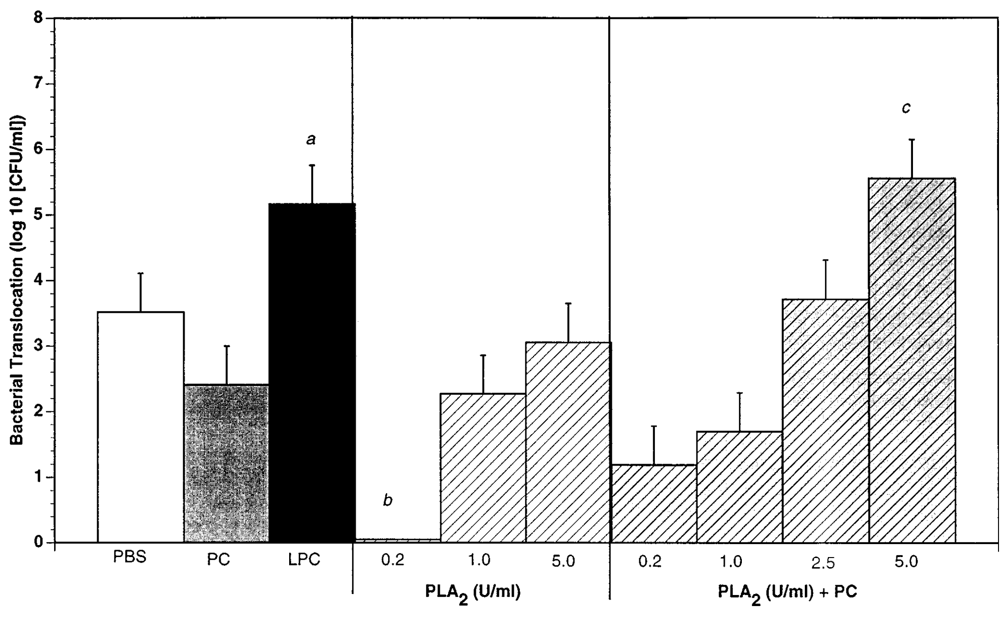

Fig. 2 Effects of phosphatidylcholine (PC), lyso-PC (L-PC), phospholipase A_2 (PLA_2) and PLA_2 + PC on bacterial translocation (BT) expressed as $\log_{10}$[CFU/ml] across Caco-2 monolayers measured 2 h after addition of PLs and/or PLA_2. (Data expressed as mean ± SD, *PBS* phosphate-buffered saline) *a* $P < 0.05$ vs PBS and PC *b* $P < 0.05$ vs PBS *c* $P > 0.05$ vs and 0.2, 1.0, 5.0 PLA_2 + PC, and PLA_2 alone and PC alone

Table 1 Effect of phosphatidylcholine (*PC*), lyso-PC (*L-PC*), Phospholipase A_2 (*PLA_2*) and PLA_2 + PC on dextran blue permeability across Caco-2 monolayers 2 h after addition of PLs and/or PLA_2. DB permeability of porous filter in absence of Caco-2 monolayer shown as control

	Dextran blue permeability (%)
No monolayer	18.2 ± 7.0
PBS	0
PC	0
LPC	0
PLA_2 (IU/ml)	
0.2	0
1.0	0
5.0	0
PLA_2 (IU/ml) + PC	
0.2	0
1.0	0
2.5	0
5.0	0

DB permeability from the apical to the basal chamber, permeability across the collagen-coated porous filters without cell monolayer was determined to be 18.7 ± 7.0% (Table 1). We found no DB permeability across control (PBS) Caco-2 monolayers or monolayers treated with PLA_2 PC, L-PC, or PLA_2 + PC.

Thin-layer chromatography

In order to confirm our hypothesis that the addition of PC as substrate to the enzyme PLA_2 would result in L-PC production in our model, TLC was performed on incubated samples taken from the apical Caco-2 monolayer surface treated under the same conditions as the TEER and BT experiments. We found that, under our experimental conditions, L-PC was generated in a PLA_2-dose-dependent manner (Fig. 3). TLC performed on samples taken from the apical surface of monolayers treated with either PC alone or PLA_2 demonstrated no L-PC production.

Discussion

The mucous layer overlying the intestinal mucosa is a component of the gut epithelial barrier and is composed of a variety of molecules including phopholipids. Utilizing a human enterocyte cell-culture model, we have previously suggested that alterations in mucous phospholipid composition may result in altered epithelial permeability and BT [12]. The intestinal enzyme principally responsible for the metabolism of luminal dietary and biliary PLs is pancreatic PLA_2; however, little information is available about mucous PL metabolism at the apical, exofacial surface of the enterocyte. Secretory PLA_2, predominantly released by Paneth cells within the small-intestinal mucosa under physiologic conditions, has been shown to markedly increase in conditions of intestinal inflammation. In addition, epithelial cells that do not typically secrete PLA_2 under normal conditions have been shown to develop significant PLA_2 immunoreactivity in regions of mucosal inflammation in patients with Crohn's disease (CD) and ulcerative colitis [1, 8]. It is known that secretory PLA_2 hydrolyzes plasma-membrane PLs to biologically-active lyso-PLs and free fatty acids, which can function as inflammatory signaling molecules. However, to our knowledge, no previous studies have addressed the potential role of mucous PL metabolism on enterocyte permeability.

In this study we have described an in-vitro enterocyte cell-culture model that has allowed investigation of the effects of extracellular PC metabolism by PLA_2 on epithelial permeability. We found that the addition of both PLA_2 and PC to the enterocyte monolayer surface resulted in the extracellular production of L-PC. L-PC, added directly or generated by PC hydrolysis, was shown to dramatically decrease the TEER across the enterocyte monolayer. The decreased TEER, measured as a function of PC plus PLA_2 treatment, was not a result of PLA_2 action on plasma membrane PLs. In fact, at a low concentration of extracellular PLA_2, TEER was slightly increased, perhaps as a result of some unestablished activity of the enzyme at the exofacial plasma-membrane surface. In addition, the effect of L-PC on

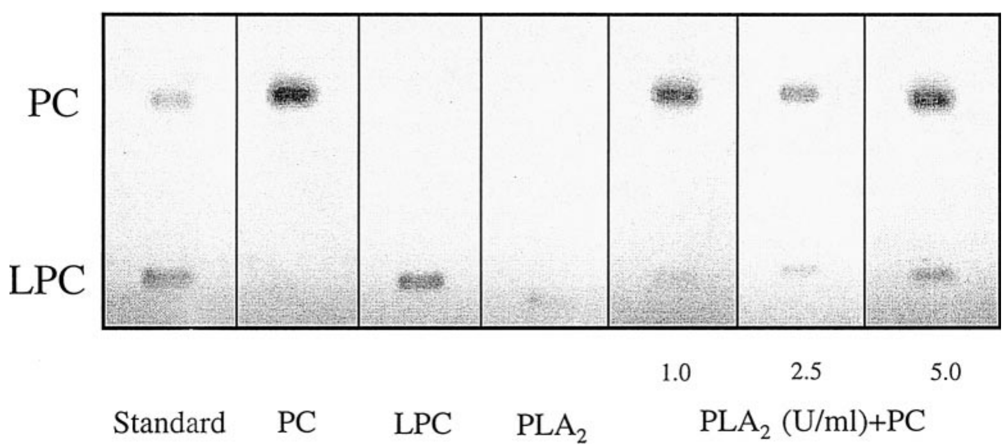

Fig. 3 Confirmation of phospholipase A_2 (*PLA_2*)-mediated hydrolysis of phosphatidylcholine (*PC*) to lyso-PC (*L-PC*) by thin-layer chromatography of samples from apical Caco-2 monolayers surface-treated with PC (2mM), L-PC (2mM), PLA_2, and PLA_2 + PC (2 mM)

TEER was not due to gross monolayer disruption as determined by the absence of DB permeability. This implies that the TEER effect of L-PC is at the level of the cellular tight junctions.

BT across the gut epithelial barrier may be involved in a wide range of inflammatory conditions such as sepsis, shock, and IBD. BT has also been extensively studied in enterocyte cell-culture models. In this report, we have demonstrated that BT is increased when enterocyte monolayers are incubated with PLA_2 and PC. This effect is a direct result of L-PC production by PLA_2 hydrolysis of extracellular PC. The addition of PLA_2 alone to the enterocyte surface did not result in increased BT, as might be expected by a direct PLA_2 effect on enterocyte plasma-membrane PL metabolism. In fact, we found a decrease in BT when monolayers were incubated with PLA_2 alone. This inhibitory effect of PLA_2 on BT may be related to the reported ability of PLA_2 to act as an antimicrobial molecule by its capacity to hydrolyze bacterial membrane phospholipids [4]. As with alterations in TEER, L-PC stimulated BT is not a result of monolayer disruption as determined by the absence of DB permeability. The effects of L-PC on TEER and BT are probably through different mechanisms in that TEER measures permeability at cellular tight junctions, while BT across intestinal epithelial cells is believed to be principally via a transcellular route involving receptor binding, engulfment, and trans-cellular transport [3].

These results suggest that in-vivo conditions in which L-PC and/or PLA_2 levels are increased within the gut lumen or at the mucosal epithelial surface may alter intestinal barrier function with increased epithelial permeability, including BT. Our model should be helpful in elucidating the cellular mechanism(s) by which L-PC, and perhaps other bioactive mucous, biliary, and dietary lipids might regulate intestinal epithelial permeability. Therapeutic strategies designed to alter dietary or mucous lipids and their metabolites may play a role in treating a variety of disorders with altered intestinal permeability such as IBD, enterocolitis, and necrotizing enterocolitis.

References

1. Almer S, Franzen L, Olaison G, Smedh K, Strom M (1991) Phospholipase A_2 activity of colonic mucosa in patients with ulcerative colitis. Digestion 50: 135–141
2. Bligh E, Dyer W (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917
3. Cruz N, Lu Q, Alvarez X, Deitch E (1994) Bacterial translocation in bacterial species dependent: results using the human Caco-2 intestinal cell line. J Trauma 36: 612–616
4. Harwig SS, Tan L, Qu XD, Cho Y, Eisenhauer PB, Lehrer RI (1995) Bacterial properties of murine intestinal phospholipids. J Clin Invest 95: 603–610
5. Kops S, Lowe D, Bement W, West A (1996) Migration of *Salmonella typhi* through intestinal epithelial monolayers: an in vitro study. Microbiol Immunol 40: 799–811
6. Minami T, Tojo H, Shinomura Y, Tarui S, Okamoto M (1992) Raised serum activity of phospholipase A_2 immunochemically related to group II enzyme in inflammatory bowel disease: its correlation with disease activity of Crohn's disease and ulcerative colitis. Gut 33: 914–921
7. Minami T, Tojo H, Shinomura Y, Komatsubara T, Matsuzawa Y, Okamoto M (1983) Elevation of phospholipase A_2 protein in sera of patients with Crohn's disease and ulcerative colitis. Am J Gastroenterol 88: 1076–1080
8. Minami T, Tojo H, Shinomura Y, Matsuzawa Y, Okamoto M (1994) Increased group II phospholipase A_2 in colonic mucosa of patients with Crohn's disease and ulcerative colitis. Gut 35: 1593–1598
9. Okuyama H, Urao M, Lee D, Abe A, Drongowski R, Harmon C, Coran A (1998) Changes, with age, in the phospholipid content of the intestinal mucous layer of the newborn rabbit. J Pediatr Surg 33: 31–38
10. Olaison G, Leandersson P, Sjeodahl R, Tagesson C (1989) Increased in permeability and phospholipase A_2 activity of colonic mucosa in Crohn's colitis. Digestion 43: 228–233
11. Stechmiller J, Treloar D, Allen N (1997) Gut dysfunction in critically ill patients: a review of the literature. Am J Crit Care 6: 204–209
12. Usui N, Ray CJ, Drongowski RA, Coran AG, Harmon CM (1999) The effect of phospholipids and mucin on bacterial internalization in an enterocyte cell culture model. Pediatr Surg Int 15: 150–154
13. Wells C, Jechorek R, Erlandsen S (1995) Inhibitory effect of bile on bacterial invasion of enterocytes: possible mechanism for increased translocation associated with obstructive jaundice. Crit Care Med 23: 301–307