Altered Glycosylation of Surfactant and Brush Border Membrane of the Small Intestine in Response to Surgical Manipulation

R. Prabhu and K. A. Balasubramanian

The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Vellore-632004, India

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Background. Surgical stress can lead to bacterial translocation from the intestine into systemic circulation. Adherence of bacteria onto the glycoconjugates of the brush border membrane (BBM) and surfactant coat (SLP) of the mucosal cells is the first step in the translocation of luminal bacteria. Our earlier study showed that surgical manipulation of the intestine results in oxidative stress leading to structural and functional alterations in the mucosa. This study looks at the effect of surgical manipulation on the glycoconjugate alterations of SLP and BBM.

Materials and methods. SLP and BBM were isolated from control and after surgical manipulation and the sugar composition was analyzed. Bacterial adherence using E. coli isolated from cecum was compared after coating microtiter plates with SLP or BBM isolated from control and after surgical manipulation.

Results. An increase in various sugars was seen after surgical manipulation both in SLP and BBM and this increase was maximum at 12 h after surgery. These alterations increased bacterial adherence onto SLP and BBM. Inhibiting superoxide generation by allopurinol treatment prior to surgical manipulation prevented glycosylation alteration and bacterial adherence.

Conclusion. Surgical manipulation results in altered glycoconjugates of SLP and BBM which leads to increased bacterial adherence. These alterations are probably brought about by oxygen-free radicals. This is clinically significant because postsurgical complications such as sepsis may be brought about by altered glycosylation.

Key Words: surfactant-like particle; brush border membrane; surgical manipulation; glycosylation; oxidative stress.

INTRODUCTION

The gastrointestinal tract is mainly involved in the digestion and absorption of nutrients. It also plays an important role in the electrolyte balance and acts as a barrier to exclude luminal contents such as bacteria and toxic substances. Intestinal mucosal cells consist of a well-defined brush border membrane (BBM) and are covered by jelly-type particles called surfactant-like particles (SLP) which separate the BBM from the luminal contents [1, 2]. SLPs are secreted initially into the basolateral space, which then migrates to the luminal surface through the tight junctions and forms a continuous coat over the brush border membrane [3]. One of the functions of this SLP is to bind luminal bacteria and thus prevent their entry into the mucosa. Glycoproteins and lipids are the important constituents of this SLP and the sugar components of this glycoprotein and glycolipids are involved in binding to bacteria. The functions of the SLP include lubrication and protection to the underlying mucosa and depend on the formation of a viscoelastic covering on the surface [4]. This in turn depends on the carbohydrate and lipid composition present on the surface of the epithelial cells. Mucosal cells produce and secrete surface-active phospholipids that share a number of biochemical and physiological properties with pulmonary surfactant [5, 6]. SLP also acts as a barrier against autodigestion and ulceration and as a vehicle for secretion of enzymes like alkaline phosphatase [7, 8].

Barrier function is also contributed by the BBM present on the epithelial cells and the barrier function can be adversely affected in pathological conditions including trauma, hemorrhagic shock, or surgical stress [9, 10]. Recent studies suggest that the intestine plays an important role in the development of postsurgical complications such as sepsis, systemic inflammatory response syndrome (SIRS), and multiple organ...
failure (MOF) [9]. Hypoperfusion is associated with physiological stress and leads to impairment of mucosal barrier function, which facilitates bacterial translocation [10]. Our earlier work has shown that surgical manipulation of the intestine can result in widening of intercellular spaces, increased intestinal permeability, and oxidative stress in the mucosa [10]. Surgical manipulation also results in structural and functional alterations in the intestinal BBM leading to altered lipid composition and sugar transport [11].

Attachment of luminal bacteria on the mucosal surface is needed for their translocation into systemic circulation and this attachment is mediated by sugars present in the glycoproteins and glycolipids. Because they are abundant in both surfactant and BBM, any alteration in their sugar composition might alter the bacterial binding [12]. The present study looks at the effect of surgical manipulation on the altered glycosylation of the intestinal SLP and BBM. Further studies were carried out on the in vitro attachment of E. coli isolated from cecal flora on the SLP and BBM obtained from control and after surgical manipulation.

MATERIALS AND METHODS

Tris(hydroxymethyl)aminomethane (Tris), bovine serum albumin (BSA), sodium metaperiodate, sodium arsenite, L-cysteine hydrochloride, thiobarbituric acid, D-mannose, D-galactose, D-fucose, glucosamine, galactosamine, Glucose oxidase, o-dianisidine, poly-L-lysine, and lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Polyethylene glycol (PEG) 4000 was obtained from Whatman No. 3 (Whatman AG Switzerland). All other chemicals used were of analytical grade.

Animals

Adult Wistar rats of both sexes (200–250 g) exposed to daily 12 h light and dark cycles and fed water and rat chow ad libitum were used for the study. The rats were randomly divided into five groups (n = 6): group I, control (laparotomy alone without intestinal handling), groups II, III, and IV different time periods such as 8, 12, and 24 h after surgical manipulation (laparotomy with intestinal handling), and group V with allopurinol treatment (100 mg/kg body weight), 1 h prior to surgical manipulation and killed 12 h after surgical manipulation. This study was approved by the Institutional Animal Experimentation Ethics Committee.

Surgical Manipulation of the Intestine

This was done as described [13]. Briefly, overnight fasted rats were anesthetized by Ketamine injection (50 mg/kg body weight, IP). The abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was gently moved and the ileocecal junction identified. The intestine was then gently handled along its entire length from the ileocecal junction proximally, simulating the “inspection” that occurs in a clinical setting. The intestine was then placed back into the abdominal cavity and the whole process was completed within 1 to 2 min. Following this, the abdominal wall was sutured and the animals were killed by decapitation, 8, 12, and 24 h after the surgical procedure. Allopurinol-treated rats were also subjected to surgical manipulation and their intestines were used for further studies. For sham control, rats were killed by decapitation after opening the abdominal wall without handling the intestine after injecting either Ketamine or allopurinol.

Isolation of Surfactant-like Particles (SLP) and Brush Border Membranes (BBM)

SLP and BBM were isolated from control and surgically manipulated rat intestine. Briefly, at indicated time periods animals were sacrificed, their abdomens were opened, and their intestines were removed and washed with ice-cold saline. SLP was isolated as described earlier [14]. Briefly, the mucosa was scraped gently with filter paper (Whatman No. 3) to remove the gelatinous SLP. The filter paper was soaked in 2 ml of PBS pH 7.4 and centrifuged at 3000 rpm for 10 min, and the supernatant was collected. The filter paper was washed twice with the same solution and centrifuged, and the supernatant collected was pooled to obtain SLP. Following removal of SLP, the underlying mucosa was used for BBM preparation. The mucosa was scraped using a glass slide and the BBM were isolated using PEG precipitation as described earlier [15]. Purity of the isolated BBM was checked by enrichment of the marker enzymes alkaline phosphatase [16], sucrase, and maltase [17]. Protein was estimated using bovine serum albumin as standard [18].

Estimation of Carbohydrate Content of the Isolated SLP and BBM

Sialic acid content was estimated as described [19]. Equal volumes of 0.2 N H2SO4 were added to the sample of SLP and BBM corresponding to a protein of 25–50 μg and digested for 1 h at 80°C. This solution was cooled to room temperature and 0.05 ml of 0.2 M sodium metaperiodate dissolved in 9 M of phosphoric acid was added and incubated for 20 min at room temperature. To this, 0.5 ml of 10% sodium arsenite in 0.5 M sodium sulfate was added and kept for 5 min at room temperature, followed by the addition of 0.75 ml of 0.6% thiobarbituric acid. This was kept for 15 min in a boiling water bath, cooled immediately, and centrifuged for 10 min; the supernatant was read at 560 nm. Standard curve was made using sialic acid in the range of 5–50 nmols and expressed as nmols/mg protein.

Hexose and fucose content of SLP and BBM were determined as described with slight modification [20]. Briefly, to 200 μl of sample corresponding to approximately 10–20 μg of protein was added 1 ml of cooled mixture consisting of six parts concentrated sulfuric acid and one part of water. This was heated for 3 min in a boiling water bath and the mixture was immediately cooled using ice. To this, 0.2 ml of CPS reagent (containing 1% of L-cysteine-HCL and 0.075% of phenol) was added. This mixture was kept in an ice bath for 60 min and the absorbance was read at 398 nm for fucose and 490 nm for hexoses. Calibration curves were made using a solution of D-galactose for hexoses or L-fucose, in the concentration range of 5–50 nmol and expressed as nmol/mg protein. Total hexosamine was estimated using Ehrlich’s reagent as described [21].

Lipid Analysis

SLP lipids were extracted by the Bligh and Dyer method [22]. The lower organic phase was evaporated to dryness, resuspended in a small volume of chloroform:methanol (2:1), and used for lipid analysis. Neutral lipids were separated on silica gel G plate using the solvent system hexane:diethyl ether:acetic acid (80:20:1, v/v/v). Spots corresponding to standard were identified by iodine exposure and eluted. Cholesterol and cholesteryl ester [23], and diacylglycerol, and triacylglycerol [24] were quantitated as described. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform:methanol:acetate water (25:14:4:2, v/v/v) and quantitated by phosphate estimation after acid hydrolysis [25].
Bacterial Adhesion Study

Coating of SLP or BBM on Microtiter Plate

Bacterial adhesion to SLP or BBM was carried out using poly-L-lysine (PLL) coated microtiter plates. For coating of microtiter plates, 10 μg PLL in PBS was added to each well and allowed to stand at room temperature for 30 min. Following this, the unbound PLL was removed from the wells, washed twice with PBS, and allowed to dry at room temperature for 1 h. These wells were then coated either with SLP or BBM. To each well, SLP or BBM corresponding to 30 μg of protein was added and allowed to stand.

FIG. 1. Sugar composition of SLP from control after surgical manipulation at various time periods. (A) Sialic acid, (B) fucose, (C) hexose, and (D) hexosamine. (Each value represents mean ± SD of four separate experiments with duplicate estimations. *P < 0.05 when compared to sham control. #P < 0.05 when compared to 12 h after surgical manipulation.)
at room temperature for 20 min. Unbound material was removed and washed twice with PBS. This resulted in coating of approximately 40–50% of the SLP and 20–30% of BBM of the added material in each well.

**Bacterial Adherence to SLP and BBM**

To each well, 0.1 ml of *Escherichia coli* isolated from cecal content of normal rats corresponding to $3 \times 10^5$ bacteria was added, and

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**FIG. 2.** Intestinal BBM sugar composition of control and after surgical manipulation. (A) Sialic acid, (B) fucose, (C) hexose, and (D) hexosamine. (Each value represents mean ± SD of four separate experiments with duplicate estimations. *P < 0.05 when compared to sham control.)
allowed to stand for 30 min at room temperature. Following this, unbound bacteria were collected and quantitated by subculturing in Macconkey plates. The number of colonies formed was counted and the percentage of bound bacteria was calculated using the following:

\[
\frac{\text{Total No. of bacteria}}{\text{unbound bacteria}} \times \frac{\text{bound bacteria}}{\text{Total No. of bacteria}} \times 100
\]

\(= \% \text{ of bacteria bound to the sample.}\)

*Escherichia coli* bound to the wells were also quantitated using crystal violet staining [26]. Following removal of the unbound bacteria, microtiter wells were washed with PBS, and 0.1 ml of 10% formalin was added and allowed to stand for 5 min at room temperature. Formalin was washed using PBS, added 0.1 ml of 1% crystal violet to each well, the dye solution removed after 3 min and the well washed twice with PBS. After drying the wells, 0.3 ml of 95% ethanol was added to each well, kept for 5 min, and read at 540 nm. An increase in absorbance (OD) indicates bacterial adherence.

**Testing of Monosaccharides as Adherence Blocking Agents**

D-mannose, D-glucose, D-galactose, D-fucose, glucosamine, and galactosamine were added to bacterial suspensions separately to obtain a final concentration of 2.5% (wt/vol), and incubated for 1 min prior to addition to SLP or BBM coated microtiter plates [27]. Quantitation of bacteria adhered to the plates was done as described above.

**Statistical Analysis**

Data are expressed as mean ± SD from a minimum of four separate preparations from different animals with duplicate estimations. Mann-Whitney nonparametric tests were used for significance of differences between groups. A probability of less than 0.05 was accepted as significant. Statistical calculations were done using SPSS (version 9) software.

**RESULTS**

Figure 1 shows various sugar contents of the isolated SLP from control and at different time periods after surgical manipulation. Increases in sialic acid, fucose, hexose, and hexosamine were seen after surgical manipulation and the maximum increase was seen at 12 h after surgical manipulation. The increase in sugar content reverted to control pattern after 24 h. Prior treat-
ment with allopurinol, a xanthine oxidase (XO) inhibitor inhibiting superoxide generation, prevented the alteration in the sugar composition of SLP following surgical manipulation. Figure 2 shows the sugar content of the BBM in control and after surgical manipulation. The alteration was similar to SLP except that maximum sialic acid was seen at 24 h after surgical manipulation, whereas all other sugars showed a maximum increase at 12 h. These alterations were not seen when the animals were treated with allopurinol prior to surgery. Figure 3 shows di- and triacylglycerol content of SLP after surgical manipulation. A decrease in

FIG. 4. SLP phospholipid composition of control and after surgical manipulation. (A) PC and lyso PC, (B) PE and lyso PE, and (C) sphingomyelin. (Each value represents mean ± SD of four separate experiments with duplicate estimations. *P < 0.05 when compared to sham control. #P < 0.05 when compared to 12 h after surgical manipulation.)
triacylglycerol (TAG) with a concomitant increase in diacylglycerol (DAG) was seen after surgical manipulation and these changes were at maximum at 12 h after surgery. Allopurinol pretreatment prevented these changes. There was no significant change in cholesterol or cholesteryl ester content of SLP after surgical manipulation (data not shown). Figure 4 shows some of the phospholipids of SLP after surgical manipulation. A decrease in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with an increase in lyso PC and lyso PE was seen after surgical manipulation and these changes were at maximum at 12 h after surgery. An increase in sphingomyelin was also seen at this time period following surgical manipulation. These phospholipid changes were also prevented by allopurinol treatment prior to surgical procedure. There was no significant change in other phospholipids (data not shown). Cholesterol/phospholipid (C/P) ratio was found to be decreased at 8 and 12 h after surgical manipulation which returned to control value by 24 h. This alteration in C/P ratio was also prevented by prior treatment with allopurinol (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
<th>12 h + allopurinol</th>
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<tr>
<td>C/P ratio</td>
<td>0.083</td>
<td>0.056*</td>
<td>0.052*</td>
<td>0.089#</td>
<td>0.08#</td>
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*P < 0.05 when compared to sham control.
#P < 0.05 when compared to 12 h after surgical manipulation.

### TABLE 1
Surfactant Cholesterol/phospholipid Ratio after Surgical Manipulation at Various Time Periods

Note. Each value represents mean ± SD of four separate experiments with triplicate estimation.

**FIG. 5.** Quantitation of bacteria bound to SLP isolated from control and after surgical manipulation at different time periods. (A) Crystal violet staining and (B) colony-forming unit (CFU). (Each value represents mean ± SD of four separate experiments with triplicate adherent study. *P < 0.05 when compared to sham control. †P < 0.05 when compared to 12 h after surgical manipulation.)
The alteration in the sugar composition might alter the bacterial adherence onto SLP which was tested by studying the interaction of *E. coli* with the SLP or BBM coated onto microtiter wells. Bacterial adherence was quantitated by the different methods, namely subculturing the unbound bacteria and by dye binding of the adherent bacteria on microtiter wells. As shown in Fig. 5, maximum bacterial binding occurred with SLP isolated at 12 h after surgical manipulation and the 24 h sample was similar to control. A similar result was obtained with BBM (Fig. 6). Allopurinol pretreatment reduced the binding of bacteria to SLP or BBM, which was similar to the control (Figs. 5 and 6). To further characterize the specificity of binding, bacteria pretreated with different sugars were allowed to bind to SLP isolated 12 h after surgical manipulation where the maximum changes in sugars were seen. It was observed that bacterial binding to SLP were inhibited to 80–90% in the presence of mannose, galactose, fucose, or galactosamine, whereas this inhibition was not seen in the presence of glucose or glucosamine (Fig. 7).

**DISCUSSION**

The intestine acts as a barrier to the luminal contents which contain bacteria and their product, endotoxin. The gut barrier can be altered in certain pathological conditions such as shock, trauma, or surgical stress, leading to bacterial or endotoxin translocation from the gut lumen into the systemic circulation [9, 28]. Under normal conditions, bacteria present in the lumen of the gastrointestinal tract remain relatively free of contact with mucosal epithelial cells as a result of a highly evolved mucosal defense system. Bacterial adherence may be considered to be pathological and this is accomplished by specific adhesions that are present on the outer surface of the bacteria and attach to the specific receptors which contain sugars such as sialic acid, hexose, fucose, and amino sugars on the surface of the epithelial cell [12, 29]. Both nonimmune (mucus, peristalsis, epithelial surfactants) and immune (secretary IgA) mechanisms normally prevent adherence of luminal bacteria on to the intestinal cells.
In certain conditions, alterations in the glycoconjugates on the surface of the cell can lead to increased bacterial adherence. Butler et al. presented evidence that the canine mucosa is covered by a nonwettable, hydrophobic layer SPL that lines the luminal surface of the epithelium [4]. SLP acts as a first defense against luminal bacteria and other pathogens. Mucous cells, particularly those on the surface epithelium, appear to be responsible for the synthesis and secretion of SLP. Maintenance of this phospholipid-rich layer on the mucosa was shown to be regulated in part by prostaglandins [31]. Bacterial adherence to the epithelial cells is sugar mediated. Several studies have shown a role for sialic acid and sugars present on mucosal surfaces acting as receptors for microorganisms [32, 33]. An increase in sialic acid, fucose, and hexosamine was seen in SLP and BBM following surgical manipulation. These alterations in the sugar composition might influence the bacterial adherence onto the epithelium. This was confirmed by bacterial adherence onto SLP and BBM coated microtiter plates. It was seen that maximum adherence was seen on SLP and BBM isolated 12 h after surgical manipulation and the changes in sugar composition of

**FIG. 7.** Inhibitory effect of various sugars on the binding of *E.coli* to SLP. (A) Crystal violet staining and (B) CFU. (Each value represents mean ± SD of four separate experiments with triplicate adherent study. *P < 0.05 when compared to 12 h after surgical manipulation.)
SLP and BBM were also maximum at this time period. This bacterial adherence was sugar specific because galactose, fucose, and galactosamine were able to inhibit this adherence, whereas glucose and glucosamine did not have any effect. Our earlier work has indicated quantitative and qualitative alterations of aerobic bacteria, especially *E. coli* in the cecal content and cecal mucosa at 12 h following surgical manipulation and reverted to control status by 24 h [34]. Studies have shown that *E. coli* adherence is mediated by the presence of fimbriae which binds to mannose containing receptors [35, 36]. Earlier work has shown that laboratory and mild intestinal handling which can occur during any abdominal surgery are capable of causing transient damage to enterocytes by activation of superoxide-generating enzyme, xanthine oxidase (XO), resulting in oxidative stress [13]. These include widening of intercellular spaces in the intestinal mucosa, permeability alterations, and decreased cellular antioxidants, as well as altered mitochondrial structure and function [37]. This results in alteration in the mucosal structure and function including increased intestinal permeability. It is likely that the changes in the sugar composition of SLP and BBM are brought about by oxygen-free radicals. This was supported by the observation that pretreatment of animals with allopurinol prevented the glycosylation alteration in SLP and BBM. These active species might modulate the activity of glycosyltransferase or glycosidases, which might alter glycosylation [38, 39]. In addition, the surface viscosity of the mucus may be altered by free radicals, which might facilitate the bacterial binding. It has been shown that viscosity of gastric mucus is markedly reduced after exposure to oxygen-free radicals [40, 41]. Alteration in the lipid composition of SLP and BBM might also influence bacterial adherence. It was seen that surgical manipulation resulted in alteration in TAG, DAG, and phospholipids, especially a decrease in PC and PE and an increase in lyso PC, lyso PE, and sphingomyeline. It was shown earlier that activation of PLA2 in the gall bladder mucosa results in the release of arachidonic acid and lysophospholipids, both of which are potent glycoconjugate secretogogues [42, 43]. Our earlier study showed that during surgical manipulation, PLA2 activation occurs, resulting in arachidonic acid and lysophospholipids formation [44], which in turn might act as glycoconjugate secretogogues.

In summary, this study has shown significant alterations in the glycosylation of SLP and BBM following surgical manipulation of the small intestine. These alterations might influence bacterial adherence onto epithelial cells, which was shown by *in vitro* adherence of *E. coli* on to SLP or BBM coated microtiter plates. It is likely that these alterations in the glycoconjugate of SLP and BBM might play a role in postsurgical complications such as sepsis.

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