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Shiga toxin exposure modulates intestinal brush border membrane functional proteins in rabbit ileum

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Abstract

The activities of lactase, sucrase and alkaline phosphatase (AP) were studied in intestinal brush border membranes of control and toxin-treated rabbits. Purified Shiga toxin (Stx) exposure to ileal mucosa inhibited activities of brush border enzymes by 50%. Kinetic analysis revealed that the observed decrease in BBM enzyme activities was due to reduced V_{max} with no change in the affinity constants of the systems. The observed changes in enzyme activities were corroborated by Western Blot analysis of lactase, sucrase and AP. The mRNA levels encoding sucrase and lactase proteins in control and Shiga toxin-treated rabbit ileum did not show any change in the rabbit ileum. Histopathological analysis showed short, blunt villi with increased number of inflammatory cells in the lamina propria and extrusion of cells in to the lumen of Stx-treated rabbit ileum. The present findings suggest that Shiga toxin act by inhibiting protein synthesis of these brush border functional proteins beyond their transcriptional level and by the direct damage to intestinal epithelium, which could be implicated in the pathogenesis of diarrhea. (Mol Cell Biochem **283:** 85–92, 2006)

Key words: brush border enzymes, intestinal malabsorption, rabbit ileum, Shiga toxin

Introduction

Bacteria colonize the intestine, invade intestinal epithelial cells and produce toxins, which play a vital role in the etiology of diarrheal diseases [1]. *S. dysenteriae1* produces a toxin known as Shiga toxin (Stx), which alters transport of Na^+ and Cl^- ions across the small intestine, and its action does not seem to involve the role of either adenylate cyclase or guanylate cyclase system [2]. The toxin acts by increasing the intracellular calcium stores, by activating protein kinases and thus phosphorylates the membrane proteins [3]. As nutrient transport is dependent upon sodium gradient across the microvillus membrane in enterocytes thus alteration in Na^+ transport may change nutrient uptake. The exposure of ileal mucosa to Shigella enterotoxin induces impairment in nutrient transport [4]. Since the molecular mechanism by which Shiga toxin modulates the expression of intestinal brush border membrane functional proteins and causes diarrhea is not completely understood, thus, Stx action on these brush border membrane (BBM) enzymes at different stages of their synthesis in rabbit ileum will be investigated.

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Materials and methods

All the chemicals used were of Molecular biology grade obtained from Sigma Chemical Co., USA and Roche Molecular Biochemicals, Germany. Antibodies against lactase, sucrase and alkaline phosphatase were a gift from Prof. D.H. Alpers Laboratory of Washington University Medical School, St. Louis, USA. The radioactive compound (γ-p^{32})-ATP was obtained from Radioisotope Division, Bhabha Atomic Research Centre, Bombay. *Shigella dysenteriae 1* strain 284 was obtained from dysentery patients attending the Nehru Hospital attached to the PGIMER and male Newzealand white rabbits were obtaind from the Central Animal House, PGIMER, Chandigarh. Before doing the experiments, animals were observed for any clinical sign of diarrhea.

Purification of Shiga toxin

The Shiga toxin was purified from the cell lysate of *S. dysenteriae1* by ammonium sulphate fractionation, dialysis, DEAE-cellulose column chromatography, followed by gel filtration on High Performance Liquid Chromatography (HPLC). The final preparation was dialysed against 0.05 M Tris-HCl (pH 8.6) and was used as the test sample. A dose standardisation of purified Stx was carried out by inoculating different amounts of toxin protein (0.5, 1.0 and 1.5 μg) into different ileal loops and fluid accumulation ratio (F.A.R.) was determined.

Rabbit ileal loop test (RILT)

The RILT was performed as described by De and Chatterjee [5]. Three adult male New Zealand white rabbits, weighing 1.5–2.0 kg were used for ileal loop surgery. Rabbits were mildly anaesthesized with sodium pentobarbital (25 mg kg^{-1}) and 2 ml of 2% Xylocaine solution was given as local anaesthesia. Hairs on belly were shaved, a middle incision was given and five ileal loops (one positive control, two negative controls and two purified Stx test samples) were constructed in one rabbit after externalizing the intestine, which was kept moist with saline. Control sample (PBS (1 ml)); positive control (cholera toxin (SIGMA), 1 μg/ml) and test sample (purified Shiga toxin: 1 μg/ml) were inoculated in different ileal loops. Each inoculated loop (7–8 cm) was separated by an uninoculated spacer interloop (2–3 cm). The animals were sacrificed after 18 h post-inoculation surgery and the fluid accumulation ratio (ml of fluid/cm of loop) was calculated. Results were recorded only for those loops where the fluid accumulation was >0.5 ml/cm of loop. Negative control loops were empty. Positive ileal loops and control intestine were removed, cleaned with chilled saline and stored at $-80\,^{\circ}$C for various biochemical analyses.

Preparation of brush border membranes (BBMs)

Microvillus membrane was isolated and purified from pooled control and toxin-treated intestines of one rabbit using the calcium chloride precipitation method [6]. Three such preparations were employed for assaying various enzymatic activities in control and toxin-treated rabbit intestine. The final membrane preparation was suspended in 50 mM sodium maleate buffer (pH 6.8) and exhibited 10–12 folds enrichment of the marker enzymes (sucrase or alkaline phosphatase) over crude homogenates. All procedures were carried out at 4 °C, except stated otherwise.

Biochemical analysis

Protein content was determined by the method of Bradford [7] using a commercial kit (Bangalore Genei) with bovine serum albumin as the standard. Lactase and sucrase activities were assayed by measuring D-glucose liberated from the respective disaccharides, using glucose oxidase peroxidase system. The method used was a modification of the procedure described by Dahlqvist [8]. Intestinal alkaline phosphatase (IAP) activity was assayed using *p*-nitrophenyl phosphate as the substrate [9]. The enzyme activities were expressed as units/mg protein. One enzyme unit is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per min under standard assay conditions.

Kinetic studies

The Kinetic studies were carried out by determining enzyme activities in the control and Shiga toxin-treated rabbit ileal brush border membranes using varying substrate concentrations: for lactase: 10, 20, 40, 80, 120 and 160 mM lactose; sucrase: 10, 20, 40, 80, 120 and 160 mM sucrose; IAP: 11.4, 22.7, 45.4, 68.5 and 90.9 mM of *p*-nitrophenyl phosphate. The data obtained were analyzed according to Lineweaver Burk transformation and Kinetic parameters (K_m and V_{max}) were calculated from the straight lines obtained by plotting $1/V$ versus $1/S$ data.

Western Blotting

Western Blot analysis was carried out as described by Towbin *et al.* [10]. Membrane protein (30 μg) was mixed with gel loading buffer (5×; 250 mM Tris-HCl, 10% β-mercaptoethanol, 10% SDS, 0.5% BPB, 50% glycerol) and were loaded on to 10% polyacrylamide gels and electrophoressed. The proteins were transblotted on to a polyvinylidenedifluoride (PVDF) membrane. After blocking

with 5% skimmed milk in PBS containing 0.1% Tween-20 (PBST), the blots were incubated for 3 h with rabbit polyclonal primary antibody (1:1000) of lactase, sucrase and alkaline phosphatase (a gift from Dr. D.H. Alpers, Washington University, St. Louis). The blots were washed in PBS containing 0.1% Tween20, incubated with goat anti-rabbit IgG secondary antibody (1:2000) and conjugated with horseradish peroxidase [Bangalore Genei].

Total RNA preparation

Total RNA from the intestine frozen in liquid N_2 was isolated following acid-guanidinium thiocynate method of Chemozynski and Sacchi [11]. Briefly, 100 mg of tissue from control and toxin-treated intestine was homogenised in acid-guanidinium thiocynate solution. The homogenate was treated with phenol-chloroform isoamyl alcohol. The RNA in the aqueous layer was precipitated by isopropanol at $-20\,^{\circ}$C and was separated by centrifugation, and dissolved in RNAase-free water.

Northern Blot analysis

Total RNA thus obtained was resolved on 1% agarose gel containing formaldehyde and ethidium bromide using 1× MOPS {3-(*N*-morpholino) propane sulphonic acid} buffer (pH 7.0). RNA samples in total volume of 25–30 μl (10 μl formamide, 5 μl formaldehyde, 3 μl 10× MOPS, 2 μl marker dye, 3–5 μl RNA sample and 2–5 μl DEPC-treated water) was mixed and denatured by boiling and chilled immediately. The mixture was then applied to the gel and electrophoressed using 1× MOPS as a running buffer [12]. The denatured RNA was transferred onto a positively charged nylon membrane (Zeta probe, Bio-Rad) by a capillary transfer method using 50 mM NaOH [13]. The transferred RNA was fixed by exposing the membrane carrying immobilized RNA to a source of low doses of ultraviolet irradiation (UV crosslinker). Prehybridization of the membrane containing immobilized RNA was carried out in SDS formamide buffer at 42 °C for 4 h [12]. Simultaneously, the [γ-p^{32}]-ATP-labelled oligonucleotide probes were added to the hybridisation bags and the sealed bags were hybridised for 20–24 h at 42 °C. After hybridisation, the membranes were washed with 2×SSC, 0.1% SDS (15 min, each three times) at 42 °C and autoradiographed by exposing the hybridized membrane to an X-ray film (Kodak XAR-2) in a cassette with an intensifying screen for 3–5 days at $-70\,^{\circ}$C. The antisense oligonucleotide probes complementary to mRNA were used. The oligonucleotide probes used were 20 bases for lactase [14], 27 bases for sucrase [15] and these probes were designed from the conservative region of c-DNA sequence already reported. β-actin probe was used as a marker of a housekeeping gene.

Histopathological analysis in Shiga toxin-treated rabbit ileum

Intestinal segments were fixed in buffered 10% formalin immediately after the animals were sacrificed. Sections 5 μm thick were cut, stained with hematoxylin and eosin. Slides were examined for any morphological changes by light microscopy.

Densitometric analysis

Densitometric scan of relative expression of different proteins by Western Blot and mRNA by Northern Blot in control and toxin-treated rabbit ileum was done by program Image J.

Results

Effect of purified Shiga toxin on brush border enzyme activities

The analysis of brush border enzyme activities revealed a decrease in sucrase ($p < 0.001$), alkaline phosphatase ($p < 0.001$) and lactase ($p < 0.05$) in rabbit ileal loops exposed to Shiga toxin compared to controls (Table 1). The observed decrease in enzyme activities was of the order of about 50% under these conditions.

Kinetic analysis of brush border enzymes

Kinetic parameters i.e. K_m and V_{max} for lactase, sucrase and AP were determined in control and toxin-treated rabbit ileum. There was no change in K_m of lactase (34.5 mM) whereas V_{max} of the enzyme was reduced from 0.18 units/mg protein

Table 1. Effect of purified Shiga toxin on brush border enzyme activities in rabbit ileum

	Control (mmoles/min/mg protein)	Toxin-treated (mmoles/min/mg protein)
Sucrase	1.95 ± 0.11	$0.98 \pm 0.084^{*}$
Lactase	0.12 ± 0.06	$0.059 \pm 0.008^{**}$
Alkaline phosphatase	2.01 ± 0.32	$0.958 \pm 0.198^{*}$

Values (units/mg protein) are mean ± S.D. of four animals.
$^{*}p < 0.001$; $^{**}p < 0.05$ vs. control using Student's '*t*' test.

in control to 0.07 units/mg protein in toxin-treated ileal loops. A decrease in the maximal velocity (V_{max}) of alkaline phosphatase from 2.63 to 1.16 units/mg protein was observed in toxin-treated ileal loops as compared to controls. However, K_m value (45.4 mM) was essentially similar under these conditions. Thus Shiga toxin exposure resulted in a decrease in V_{max} of lactase and AP with no effect on the affinity constant of enzymes. Brush border sucrase kinetic parameters were also evaluated on the same BBM preparation as for lactase and AP. K_m of sucrase (29.8 mM) remained unaltered in control and toxin-treated intestine. But again the V_{max} of the enzyme was reduced from 2.85 in control to 1.53 units/mg protein in toxin-treated rabbit intestine.

Western Blotting

The observed changes in brush border enzyme activities in toxin-treated rabbit ileal loops were further analyzed by Western Blot analysis using polyclonal antisera against these enzyme proteins. For these experiments, 30 μg of membrane proteins were separated on the acrylamide gels by SDS-PAGE and transferred on to PVDF membranes. The immunoblot for lactase revealed two distinct bands of 220 kDa and 130 kDa proteins in the control and toxin-treated rabbit ileum preceding the expression of prolactase and mature lactase, respectively (Fig. 1A). The intensity of two protein bands was significantly reduced in Shiga toxin-treated rabbit intestine compared to control loops as evidenced by densitometric analysis (Fig. 1B).

Immunoblot for brush border sucrase (Fig. 2A) exhibited a single band corresponding to 210 kDa protein in control rabbit intestine. The band intensity was drastically reduced in ileum exposed to the toxin as compared to control. This is apparent in densitometric scan of the data shown in Fig. 2B. Analysis of alkaline phosphatase protein by immunostaining also revealed the similar results. A distinct band of 66 kDa protein was observed in the control tissue whereas a relatively faint protein band was observed in Shiga toxin-treated rabbit ileum at the same position (Fig. 3A).

Northern Blot analysis of brush border enzymes expression

Total RNA was extracted from control and toxin-treated rabbit intestine and was resolved on 1% agarose/formaldehyde gel. Two distinct RNA bands were visible in the agarose gel under ultraviolet light. These bands corresponded to two major ribosomal RNAs (rRNAs) viz. 28S and 18S (Fig. 4). The RNA was transferred on to positively charged nylon membrane and hybridised with [γ-^{32}P]-ATP-labeled specific oligonucleotide probes of the respective enzymes. Hybridization using [γ-^{32}P]-ATP labelled β-actin oligonucleotide probe was also carried out. As shown in Fig. 5A

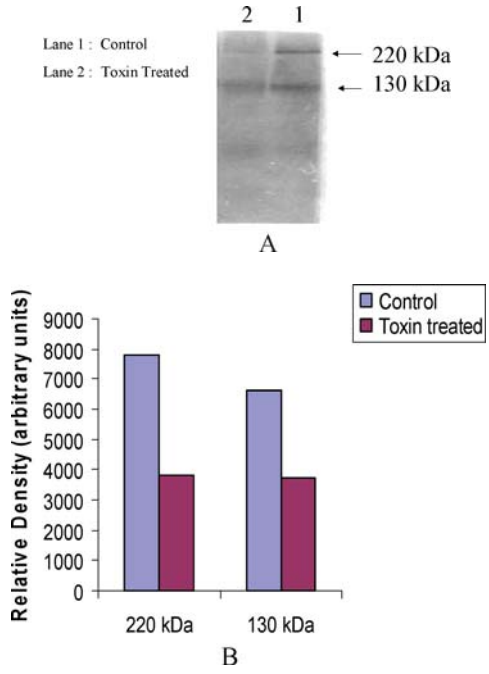

Fig. 1. (A) Western Blot analysis of brush border lactase in control and purified Shiga toxin-treated rabbit ileum. Polyclonal antisera against intestinal lactase (1:1000) followed by secondary antibody (1:2000) were used. Colour was developed using TMB/H_2O_2 as a substrate solution. (B) Densitometric scan of data given in (A).

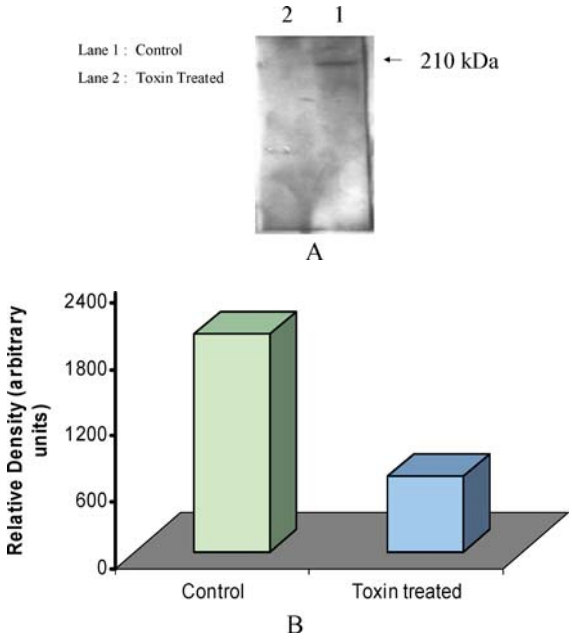

Fig. 2. (A) Western Blot analysis of brush border sucrase in control and purified Shiga toxin-treated rabbit ileum. Polyclonal antisera against intestinal sucrase (1:1000) followed by secondary antibody (1:2000) were used. Colour was developed using TMB/H_2O_2 as a substrate solution. (B) Densitometric scan of data given in (A).

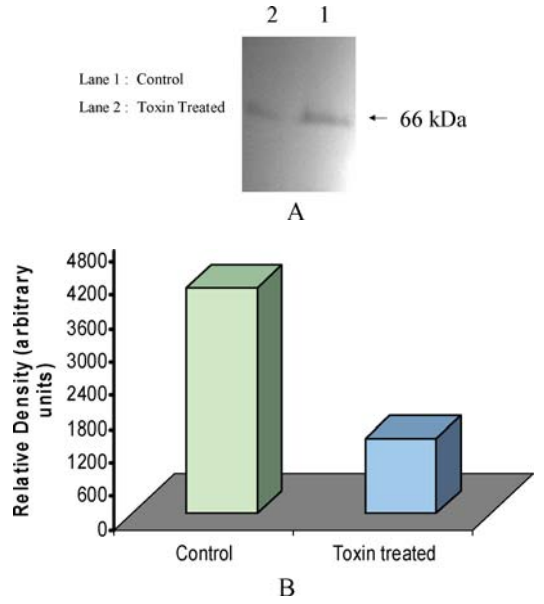

Fig. 3. (A) Western Blot analysis of brush border alkaline phosphatase in control and purified Shiga toxin-treated rabbit ileum. Polyclonal antisera against intestinal alkaline phosphatase (1:1000) followed by secondary antibody (1:2000) were used. Colour was developed using TMB/H_2O_2 as a substrate solution. (B) Densitometric scan of data given in (A).

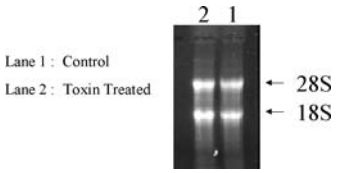

Fig. 4. Total RNA from control and Shiga toxin-treated rabbit ileum was obtained by guanidinium thiocynate and phenol-chloroform extraction, analysed on 1% agarose gels stained with ethidium bromide.

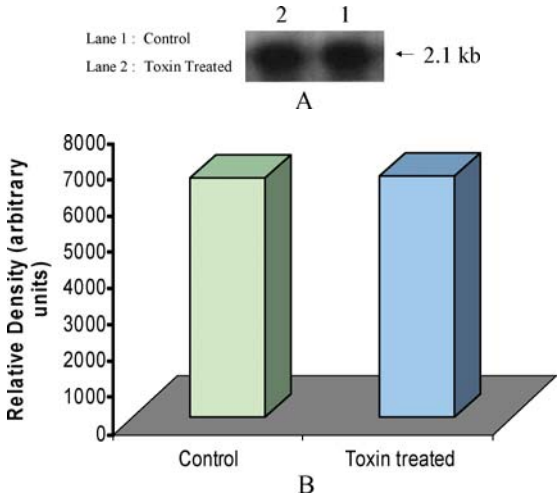

Fig. 5. (A) Northern Blot analysis of mRNA encoding β-actin in control and toxin-treated rabbit intestinal tissue. Each lane was loaded with 10 μg of total RNA. Blots were hybridised with radiolabelled oligonucleotide probes. Densitometric scanning of β-actin mRNA expression is in Fig. 5 (B).

there was no change in mRNA levels encoding β-actin gene (2.1 kb), used as an internal marker of housekeeping gene in the control and toxin-treated animals. Densitometric scan of β-actin blot is shown in Fig. 5B. The autoradiographs of lactase showed a very distinct band of 6.8 kb mRNA encoding lactase (Fig. 6A). However, mRNA levels

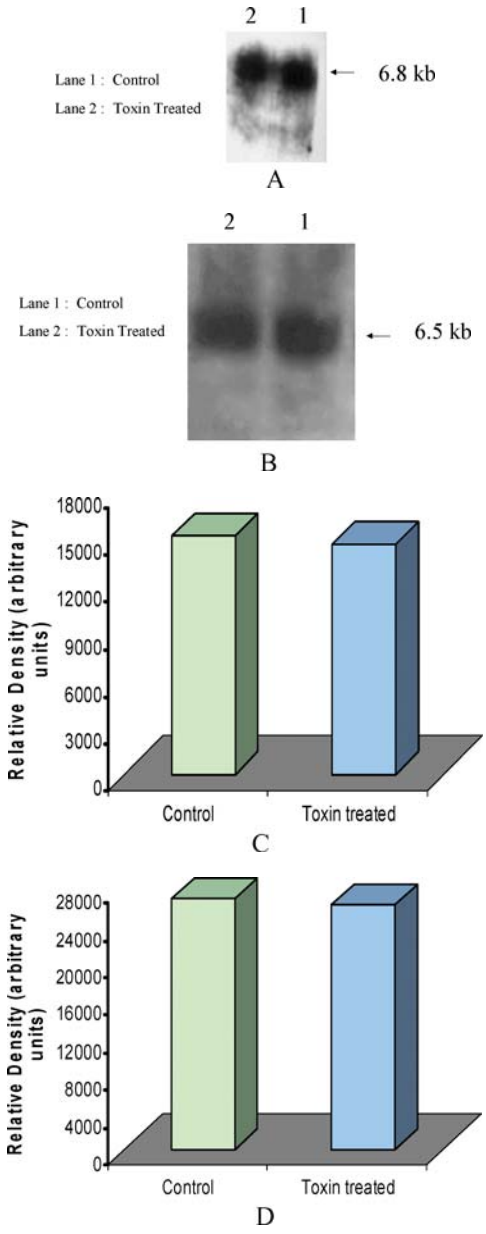

Fig. 6. Northern Blot analysis of mRNA encoding brush border lactase (A) and sucrase (B) in control and toxin-treated tissues. Each lane contained 10 μg of intestinal RNA. Blots were hybridised with radiolabelled oligonucleotide probes. Autoradiography was carried out using X-ray film (Kodak XAR-2) and intensifying screens at $-70\,^{\circ}$C for 3–5 days. Densitometric scanning of lactase and sucrase mRNA expression is in Figs. C and D, respectively.

encoding brush border lactase in control and toxin-treated intestinal tissue did not show a significant difference under these conditions. Similarly, autoradiographs of sucrase exhibited 6.5 kb mRNA transcript (Fig. 6B), but intensity of mRNA bands encoding sucrase in control and toxin-treated tissues reflected no significant difference which suggested that mRNA expression of these brush border enzymes in control and toxin-treated ileum, are not affected by toxin treatment. These results are apparent in densitometric scan of lactase and sucrase mRNA expression in Fig. 6C and D, respectively.

Histopathological analysis in Shiga toxin-treated rabbit ileum

Histopathological analysis of normal rabbit intestine under light microscopy using Hematoxylin and Eiosin (H and E) staining revealed that the wall of the intestine consists of few layers from interior to exterior side i.e. mucosa, submucosa, muscular coat and serosa. Villi with characteristic everted folds of the mucosa were noticeable (Fig. 7A). Purified Shiga toxin-treated rabbit intestine showed blunt and short villi. The architecture of the villus was altered, particularly near the tip

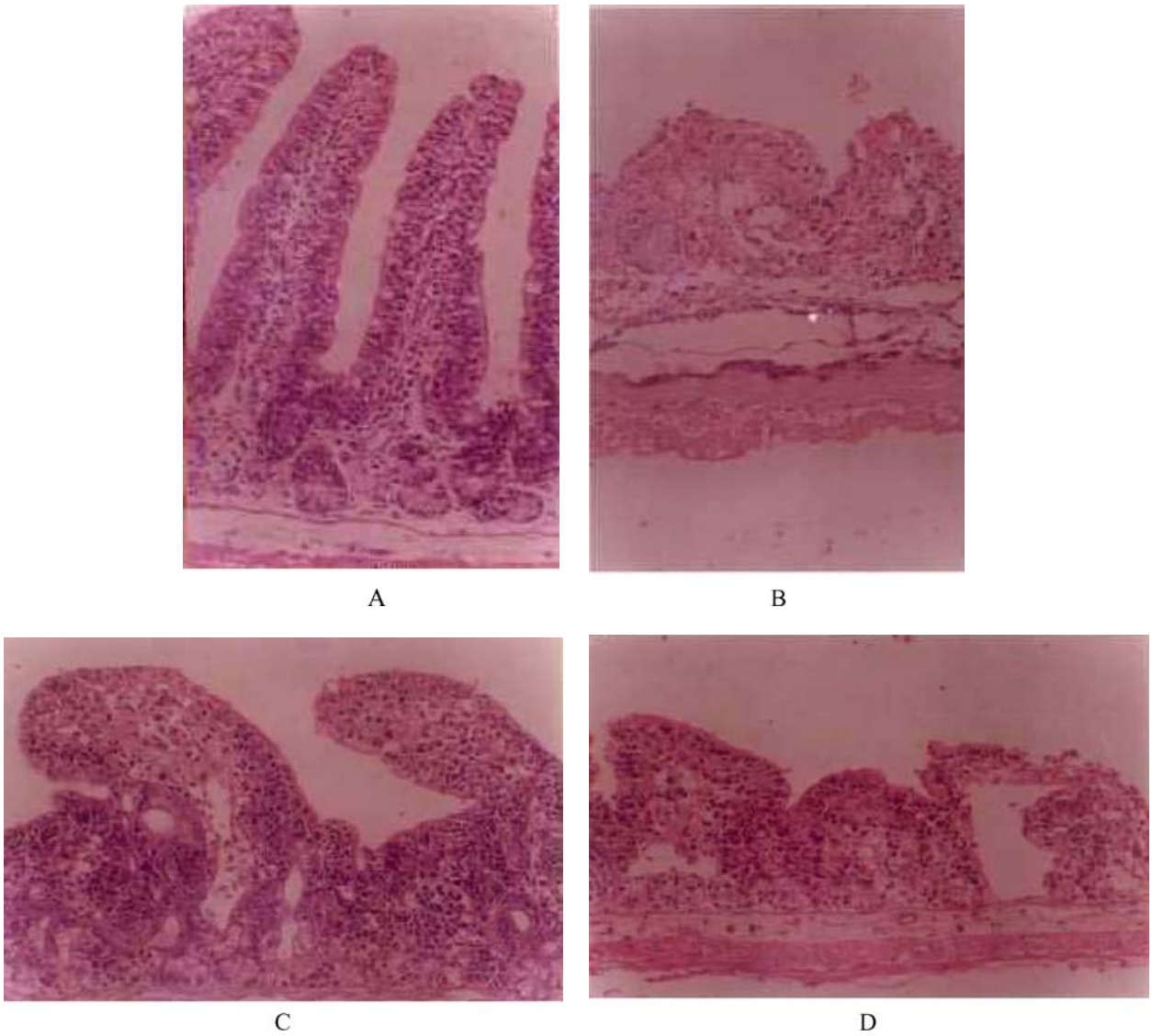

Fig. 7. (A) Normal rabbit ileal mucosa (Hematoxylin and Eosin, 20×), (B, C, D) Rabbit ileal mucosa after challenge with purified Shiga toxin (Hematoxylin and Eiosin, 20×).

where the epithelium was flattened and replaced in part by a cap of altered epithelial cells. Individual epithelial cells or small clumps of altered cells were extruding into the lumen from the side as well as from the tip of the villus (Fig. 7B). Acute inflammatory cells were increased in number in lamina propria and in the epithelium (Fig. 7C). Stx exposure resulted in dilation of vascular spaces in lamina propria and formation of spaces due to necrosis of cells in rabbit ileum (Fig. 7D).

Discussion

The brush border enzymes are not only important in digestion and absorption of the nutrients from the intestinal lumen but are also important for the cellular activities of the enterocytes. Any change in the activities of these enzymes during parasitic infection may reflect physical damage to the enterocytes which may in turn depict the altered functional or cellular responses of the cells. The activities of disaccharidases and AP were markedly reduced in toxin-treated animals. The observed decrease in enzyme activities was a consequence of reduced maximal velocity (V_{max}) without affecting the affinity constant (K_m) of the enzymes. These observations were further analysed by Western Blot performed in infected and control tissues. These findings revealed low amounts of the enzyme protein in toxin-treated intestinal tissue, compared to controls. However, Northern Blot analysis did not show any change in the mRNA levels encoding disaccharidases in Shiga toxin-treated intestinal tissue. This also suggests that the expression of these proteins at the transcriptional level is not effected under these conditions. Thus the effect of toxin in alteration of protein level as suggested by Western Blot analysis may presumably be at the post-transcriptional level (translational or post- translational level) of brush border functional proteins. These results are not surprising in view of previous studies of Shiga toxin reported in literature in relation to its mode of action. Shiga toxin binds to cell surface receptor and is subsequently internalised, presumably through receptor-mediated endocytosis in order to reach to its ribosomal site of action [16]. Shiga toxin like other plant (ricin, abrin, modeccin) and fungal (α-sarcin) toxins has RNA-N glycosidase activity and this activity may be responsible for ribosome inactivation leading to inhibition of protein synthesis [17–19]. Toxin-induced protein synthesis inhibition in rabbit jejunum villus cells has also been reported by Kandel *et al*. [20]. This inactivation process seems to be a general mechanism for ribosome inactivating cytotoxins and it provides additional evidence of the cytotoxic effect of Shiga toxin which is well documented in literature.

Intolerance to lactose and other disaccharides is frequently observed in association with acute diarrhea [4]. Broitmen [21] also reported a decrease in intestinal lactase activity during experimental Salmonellosis and one recent report from our laboratory showed decreased glucose transport as well as decreased brush border enzyme activities in *Salmonella typhimurium* infected rabbit ileum [22]. Purified Shiga toxin is capable of causing acute enteritis in the rabbit ileum. The histological characteristics after Shiga toxin treatment are shortening and broadening of villi, altered villus architecture, flattened epithelium, and acute inflammatory cells increase in number in lamina propria which are similar to those reported by Keush *et al*. [23], whereas there is a single contrasting report of no inflammation or mucosal injury [24] and showed that Stx induces secretion in the absence of histological changes. It has been reported in literature that Shiga and Shiga-like toxins act directly and selectively on mature columnar absorptive epithelium of intestinal villus resulting in premature expulsion of these cells from the villus wall, with a decrease in villus/crypt ratio [25].

A diffuse decrease in brush border enzyme activities and transport function in the small intestine with villus atrophy and crypt hyperplasia in the ileum has been reported in acute *Yersinia enterocolitica* infection [26]. Another study reported that infection of weanling rabbits with *Yersinia enterocolitica* causes diarrhea and decreased disaccharidase activity in all regions of the small intestine [27]. The morphological and mucosal enzyme alterations are due to intestinal injury by the organism. Thus in the present study, following mechanisms can be proposed for the observed decrease in BBM enzyme activities due to Stx toxicity: sloughing or extrusion of some of the villus tip cells, protein synthesis inhibition in the cytoplasm, rapid degradation of translated proteins and defective insertion of mature proteins into the microvillus membrane (MVM). Parellelising above proposed mechanisms with the so far reported literature of Stx action on intestinal epithelium it's concluded that the observed decrease in BBM enzyme proteins may be the combined effect of Stx on protein synthesis inhibition deep inside the cells, followed by detachment of cells from the villus surface along with its direct damage to the epithelium. A similar mechanism has been reported by Obrig *et al*. [28] that Stx inhibits protein synthesis-preceded detachment of cells from the substratum which may result in expulsion of these cells from the villus wall, resulting in decrease in the villus/crypt ratio in human endothelial cells. Thus, the present findings demonstrate that enterotoxin produced by *Shigella dysenteriae1* modulates the molecular mechanism of BBM functional protein expression in rabbit ileum, which may be associated with intestinal malabsorption observed in this infection.

Acknowledgments

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