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Characterization of the interaction of *Escherichia coli* heat-stable enterotoxin (STa) with its putative receptor on the intestinal tract of newborn calves

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) induces severe diarrhea in newborn calves through the elaboration of heat-stable enterotoxin (STa). We investigated the distribution and characteristics of the STa-specific receptors on enterocytes and brush border membrane vesicles (BBMVs) prepared from anterior jejunum, posterior jejunum, ileum and colon of newborn calves. We found that density of the receptors and their affinity to STa were higher on enterocytes and BBMVs that were derived from the ileum than enterocytes and BBMVs prepared from other segments of the calf intestine. This study suggests that, in newborn calves, the ileum is the major part of the intestinal tract that is affected in the course of secretory diarrhea caused by STa-producing ETEC strains.

Keywords: Heat-stable enterotoxin ; Receptor; Newborn calf; *Escherichia coli*

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of colibacillosis in newborn calves that is characterized by profuse yellow-white fluid diarrhea, dehydration and depression [1–3]. To cause diarrhea1 disease in newborn calves, *E. coli* must be able to adhere to the intestinal mucosa and elaborate enterotoxins [1–5]. Secretory diarrhea caused by infection with ETEC has been shown to be mediated by two classes of enterotoxins; the heat-labile enterotoxin (LT), which has been characterized and found to be closely related to cholera toxin functionally and structurally, and the heat-stable enterotoxin (ST) [1,6–8]. There are two subtypes of ST: STa and STb. STa is associated with disease in both humans and animals, and STb is associated primary with diarrhea in piglets [8]. Bovine ETECs typically produce only STa [1,2,9, 10]. This toxin was found to cause fluid distention in ligated calf intestine [1 1] and its action can be demonstrated in infant mice [12,13]. STa has a small molecular mass (2 kDa) and is poorly immunogenic [8], therefore, immunity is not expected to play a role in the protection of newborn calves against this diarrhea1 disease. Although slight variations in the size of STs were observed in some host-specific ETEC strains, no difference in the mechanism of STs action nor their specific activity was observed. [14–16]. Strains of bovine ETEC may vary considerably with respect to the amount of STa produced in vitro [17].

STa receptors are found throughout the small intestine and the colon [8,18]. It is believed that STa receptors which are present on the intestinal epithelium of newborn animals rapidly decrease with increasing age [4,19,20]. However, these aspects were not investigated in calves. Using a suckling mouse model, we found that the affinity STa receptors are age-dependent [20]. Studies performed on mammalian cell lines suggested that STa receptor is an extracellular domain of guanylate cyclase (guanylate cyclase type C or CC-C) which is located on the apical membrane of intestinal epithelial [21,22]. GC-C that has a molecular mass of 120 kDa is one of a family of receptor cyclases that include the atria1 natriuretic peptide recep-

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tors, GC-A and GC-B [23,24]. Upon binding its receptor, STa activates GC, which converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP) [15,25,26]. Increases in intracellular cGMP inhibit the Na^+/Cl^- co-transport system in the small intestinal villus enterocytes [2,27].

The purpose of this study was to characterize the interaction of *E. coli* STa with its putative receptor on newborn calf intestinal tract to determine the receptor distribution, its affinity to ST and its density on enterocytes and brush border membrane vesicles (BBMVs) prepared from different segments of calf small intestine and colon.

2. Materials and methods

2.1. Experimental animals

Six male 1-4-day-old colostrum-fed Holestein calves were obtained from Purdue Animal Research Farm. Calves were killed by intravenous injection of Beuthonasia-D. The abdominal cavity was opened and the intestine was removed. The jejunum was divided into two parts; anterior and posterior. Segments of equal length from the anterior jejunum, posterior jejunum, ileum and colon were used for the preparation of enterocytes and BBMVs.

2.2. Isolation of enterocytes from calf intestine

From each intestinal segment, 20 cm was thoroughly washed with ice-cold phosphate-buffered saline (PBS). Segments were incubated on ice for 2 h in ice-cold PBS solution that contained 1.5 mM EDTA and 0.5 mM dithiothreitol. Segments were gently agitated every 15 min. Enterocytes were harvested by centrifugation at 1000 **x** g for 15 min. Cells were resuspended in PBS containing 1% bovine serum albumin (BSA). Enterocyte counts and viability were determined after exclusion of the 0.2% trypan blue-stained enterocytes by microscopic examination. Dry smears were prepared for indirect immunofluorescence analysis. Enterocyte populations with > 70% viable cells were used for flow cytometry and [^{125}I]STa binding assays.

2.3. Preparation of the BBMVs

The intestinal mucosa was scraped off with an edge of a glass slide, and the scrapings were pooled and frozen at -70°C. When needed, scrapings were thawed, weighed and resuspended with homogenate medium (50 mM mannitol, 2 mM Tris-HCl, pH 7.4) at a ratio of 20 ml of homogenate media per gram of mucosal scrapings. The scrapings were homogenized for 60 s at setting 3 of the Omni-2000 homogenizer (Omni Int'l, CT, USA) and centrifuged for 15 min at 200 **Xg.** $MgCl_2$ was added to the supernatant to give a final concentration of 10 mM, and the supernatant was stirred in the cold room for 15 min and then centrifuged for 15 min at 2400 $\times g$ to pellet the precipitate. The resultant supernatant was centrifuged for 30 min at 19 000 **x** g to generate the crude BBMV pellets. The supernatant was poured off, and a total of 6 ml of resuspension medium (300 mM mannitol, 50 mM HEPES-Tris, pH 7.0) was added to the centrifuge tubes. The pellets were resuspended by repeated passage through a 26-gauge needle, pooled, divided into 0.25-ml aliquots and frozen at 120°C until the day of use. For a given experiment, a suitable number of aliquots of crude BBMVs were thawed in 70 ml of the resuspension media required for the particular experiment and homogenized in a glass-Teflon homogenizer (six strokes) prior to centrifugation for 15 min at 500 **Xg.** The resultant supernatant was centrifuged for 30 min at 39 000 $\times g$ to generate the final BBMVs pellets. The final pellets were resuspended with a 26-gauge needle, and a suitable volume of resuspension media was pooled, assayed for protein content and diluted to give a vesicle concentration of 16 mg protein ml-'. Electron microscopic examination of the BBMVs suspension revealed homogenous vesicular structures.

2.4. Indirect immunofluorescence

Enterocytes from the different segments of calf intestine were processed for indirect immunofluorescence as previously described [20,28].

2.5. Flow cytometry

Enterocytes isolated from different segments of calf intestine were processed for flow cytometry as described previously [20]. BBMVs were processed for flow cytometry similar to enterocytes with slight modifications. Frozen samples of BBMVs were thawed and suspended in 10 ml of resuspension media. BBMVs were washed twice with PBS by centrifugation at 19 000 **x** g for 30 min. BBMV suspensions were incubated with 50 μl of HPLC-STa at 37°C for 45 min. After washing with PBS, BBMVs were incubated at 4°C for 30 min with 50 μl of 1: 10 diluted STa-specific antiserum. Fifty microliter of goat anti-rabbit serum, FITC-conjugated (KPL, Gaithersburg, MD, USA) diluted 1: 100 in PBS was added to the BBMV suspensions and incubated for 30 min on ice. BBMVs were then washed three times with PBS-BSA, resuspended in 1.0 ml of PBS and kept on ice until flow cytometric analysis was performed. As negative controls, similar samples were incubated with STa and anti-rabbit IgG FITC-conjugated antibody and used to determine the threshold of specific staining. Isotope controls were used to control non-specific fluorescence. Flow cytometric analysis was preformed using Epics ELITE flow cytometer (Coulter Electronics, Hileah, FL, USA). The flow cytometer was set to read 10 000 cells from each enterocyte preparation. FITC-stained cells

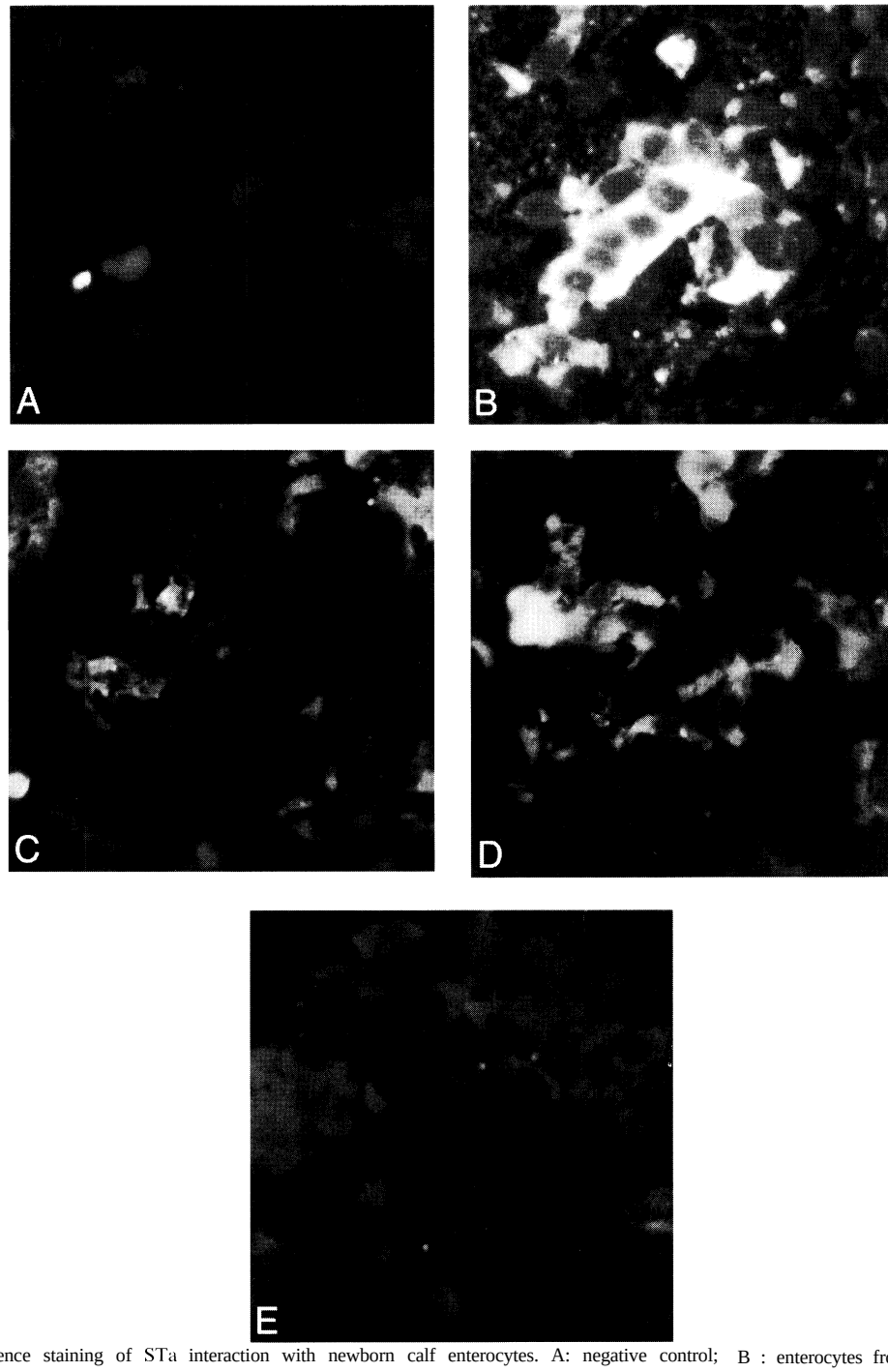

Fig. 1. Immunofluorescence staining of STa interaction with newborn calf enterocytes. A: negative control; B : enterocytes from ileum; C: enterocytes from anterior jejunum ; D : enterocytes from posterior jejunum ; E: enterocytes from colon. All enterocytes were stained sequentially with STa, anti-STa rabbit serum and anti-rabbit IgG FITC antibodies except for the negative control where no STa was used.

were excited by using 15 mW of 488-nm argon laser light. FITC-conjugated beads were run and the mean fluorescent intensity was set at a fixed value.

2.6. Iodination of heat-stable enterotoxin (STa)

One hundred microgram of HPLC-STa was iodinated following a protocol reported by Al-Majali et al. [28]. The protein concentration was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The purified radiolabeled STa had a 350-mCi nM^{-1} specific activity.

2.7. Radiolabeled STa binding assay

Reaction mixtures containing calf enterocytes (1×10^4) or BBMVs (16 μg), PBS-BSA and [^{125}I]STa (20-640 nM)) were incubated in a final volume of 200 μl for 40 min at t

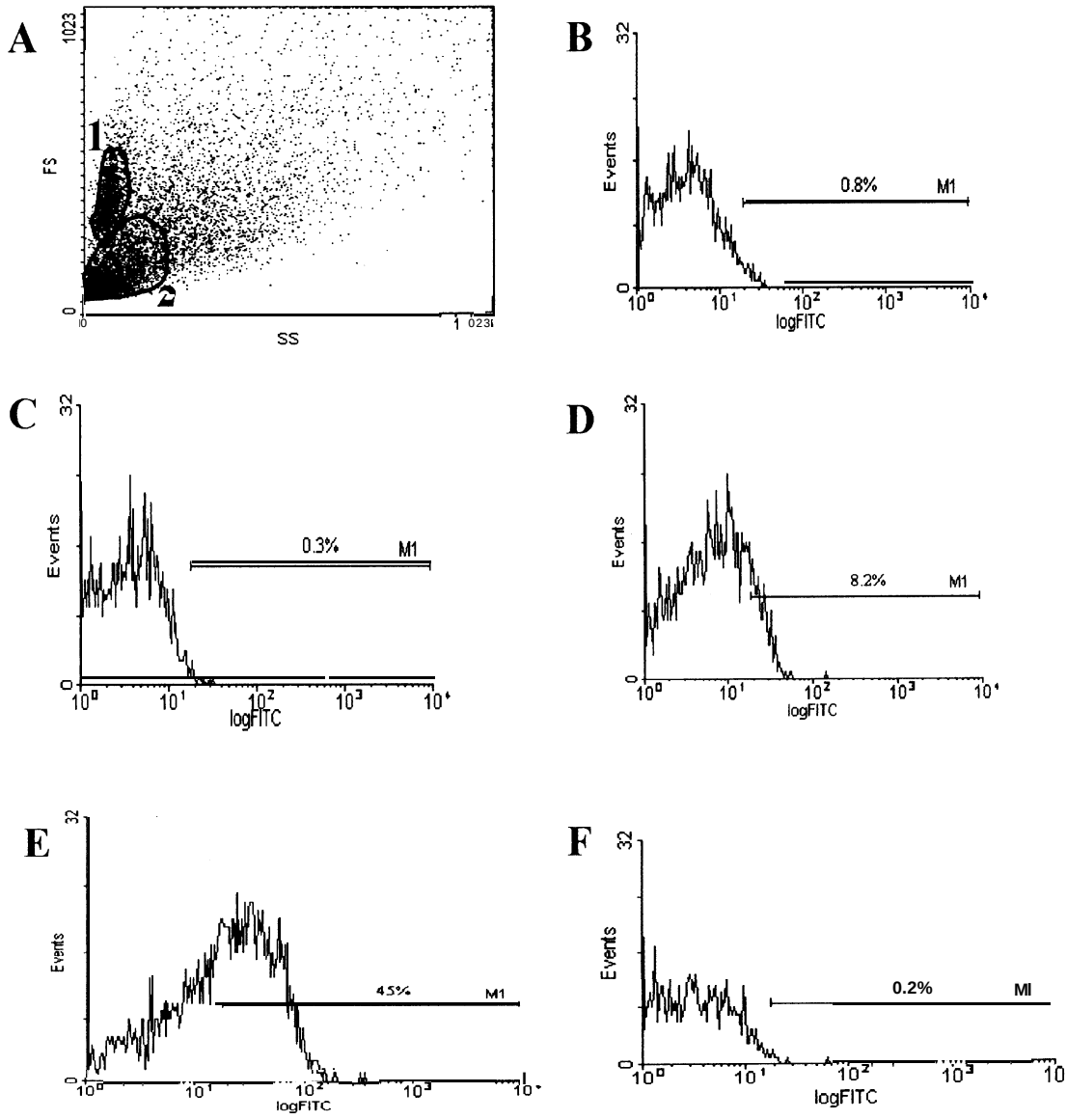

Fig. 2. Representative flow cytometry histograms for STa interaction with enterocytes from different segments of newborn calf intestinal tract. A: forward scatter versus side scatter for all enterocytes tested; B : negative control; C: enterocytes from anterior jejunum; D: enterocytes from posterior jejunum ; E : enterocytes from ileum ; F: enterocytes from colon. All enterocytes were stained sequentially with STa, anti-STa rabbit serum and anti-rabbit IgG FITC antibodies except for the negative control where no STa was used. Gate 2 shown in A is used to generate the profiles shown in B, C, D, E and F.

37°C in a shaking water bath. Unbound [^{125}I]STa was removed from bound [^{125}I]STa by vacuum filtration (Millipore, Bedford, MA, USA), using 1-μm, 2.5-cm GF/B glass filters (Whatman, Maidstone, UK). Total binding was measured in a reaction mixture that did not contain the unlabeled STa, whereas non-specific binding was measured in a reaction mixture that contained the labeled STa including 1000-fold excess of unlabeled STa. Specific binding was calculated by subtracting non-specific binding from the total binding. Scatchard plots for the [^{125}I]STa-specific binding data were constructed by plotting the bound [^{125}I]STa against the ratio between the bound and the free [^{125}I]STa. The dissociation constant (K_d) and the maximum number of STa receptors (B_{max}) were calculated using the Rosenthal-Scatchard equation [29,30].

3. Results

3.1. Indirect immunofluorescence assay

Indirect immunofluorescence study of enterocytes from the different intestinal segments of newborn calves revealed the localization of intensely stained areas concentrated mostly at the brush border membrane regions. Enterocytes isolated from the ileum showed the most

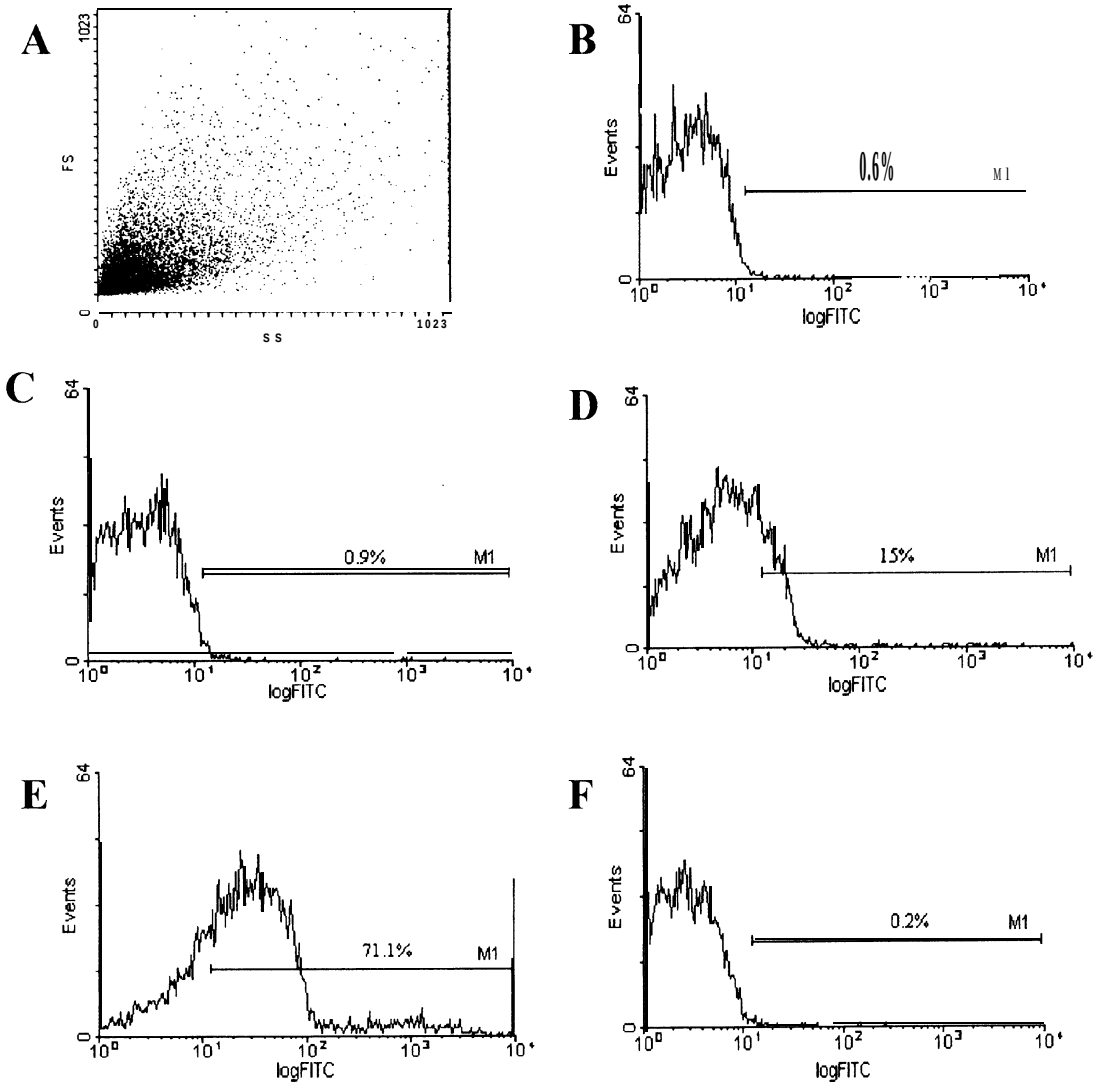

Fig. 3. Representative flow cytometry histograms for STa interaction with BBMVs prepared from different segments of newborn calf intestinal tract. A: forward scatter vs. side scatter for the BBMVs population; B: negative control; C: BBMVs from anterior jejunum; D: BBMVs from posterior jejunum; E: BBMVs from ileum; F: BBMVs from colon. All samples were stained sequentially with STa, anti-STa rabbit serum and anti-rabbit IgG FITC antibodies except for the negative control where no STa was used.

intensely fluorescent brush border membranes (Fig. 1). Fluorescence intensities were relatively low on enterocytes isolated from anterior jejunum, posterior jejunum and colon. The absence of fluorescence on the negative control smears suggests that the interaction between STa and its receptor was specific.

3.2. Flow cytometry

Two populations of enterocytes that differ in their forward scatter values were observed. The enterocyte population with the high forward scatter value represented only 10–20% of the total enterocytes analyzed by flow cytometry (Fig. 2A). The fluorescence level associated with enterocytes isolated from the ileum was higher than the fluorescence level associated with enterocytes from other segments of the calf intestine (Fig. 2B–F). Stronger fluorescence signals were detected on the posterior jejunum compared to that of the anterior jejunum and colon. Signals on enterocytes from the anterior jejunum and colon were similar to that of the negative control suggesting the absence of the receptors for STa on brush border membranes of enterocytes extracted from these parts. Similar results were obtained upon analyzing stained BBMVs from the different segments of the calf intestine (Fig. 3B–F). Unlike the stained enterocytes, forward scatter analysis of BBMVs prepared from the different segments of the calf intestine suggested the existence of one population of these vesicles (Fig. 3A).

3.3. Stoichiometry of STa receptor in the different section of newborn calf intestine

Binding of [^{125}I]STa to enterocytes and BBMVs pre-

Table I
Binding properties of [^{125}I]STa to enterocytes and BBMVs prepared from different segments of newborn calf intestine

Intestinal segment	Specific binding (mean ± S.D.)		Non-specific binding (mean ± S.D.)		Dissociation constant (K_d) (mean ± S.D.)		STa receptor (mean ± S.D.)	
	Enterocytes (%)	BBMVs (%)	Enterocytes (%)	BBMVs (%)	Enterocytes (nM)	BBMVs (nM)	Enterocytes (receptors per enterocyte)	BBMVs (nM mg^{-1} protein)
Ileum	87 ± 5.3[a]	81 ± 3.4[a]	13 ± 5.3[a]	19 ± 3.4[a]	10.9 ± 1.4[a]	9.3 ± 1.6[a]	16 171.1 ± 3 367.2[a]	15.7 ± 1.9[a]
Anterior jejunum	32 ± 5.7	21 ± 7.2	68 ± 5.7	79 ± 7.2	77.0 ± 9.0	96.3 ± 8.9	611.2 ± 93.8	1.8 ± 0.2
Posterior jejunum	48 ± 6.2	42 ± 6.2	52 ± 6.2	58 ± 6.2	26.2 ± 7.8	55.8 ± 11.1	3 715.5 ± 710.1	5.0 ± 0.9
Colon	19 ± 3.0	9 ± 1.7	81 ± 3.0	91 ± 1.7	105.9 ± 20.8	150.1 ± 35.8	248.8 ± 62.4	1.2 ± 0.2

[a]The differences between the ileum and other intestinal segments were statistically significant ($P < 0.01$) using one-way ANOVA followed by pail-wise comparison probabilities (Bonferroni correction).

pared from different segments of the calf intestine was saturable and reached a plateau. The specific binding of [^{125}I]STa to enterocytes and BBMVs prepared from the ileum was higher than that of enterocytes and BBMVs prepared from other segments of the calf intestine (Table 1). Scatchard analysis of the specific binding data suggested the existence of a single class of STa receptor on enterocytes and BBMVs of different segments of the calf intestine. Both density and affinity of these receptors to STa were high in enterocytes and BBMVs prepared from the ileum. STa receptor densities on enterocytes (1.6×10^5 per enterocyte) and BBMVs (15.66 nM mg^{-1} protein) prepared from the ileum were about 4–65-fold higher than that of enterocytes and BBMVs prepared from other parts of the calf intestine (Table 1). In addition, STa receptor affinities on enterocytes (10.92 nM) and BBMVs (9.333 nM) isolated from the ileum were about 3–16-fold higher than that of enterocytes and BBMVs isolated from other parts of the calf intestine (Table 1). The values of STa receptor affinities and densities of enterocytes and BBMVs prepared from the posterior jejunum were higher that that of the anterior jejunum and colon. The differences in the affinity and density of STa receptor on enterocytes and BBMVs isolated from the different segments of newborn calves intestine were statistically significant as suggested by pairwise Student's t-test and multiple comparison group analyses (Bonferroni correction).

4. Discussion

Colibacillosis in newborn calves is a very serious disease during the first 48 h of life. A critical step in the pathogenesis of this disease is the binding of STa to its putative receptor on the brush border membrane of intestinal epithelium. Upon STa binding, an impairment in the Na^+/Cl^- transporting mechanism occurs and a secretory diarrhea will develop. In this study, we investigated the distribution of STa receptor throughout the intestine of newborn calves. Indirect immunofluorescence and [^{125}I]STa binding assays were used to characterize the affinity and density of this receptor along the intestinal tract of newborn calves.

ETEC STa receptors were found on the brush border membranes of enterocytes throughout the intestinal tract of newborn calves. Receptors were only found on the apical surfaces of these enterocytes. STa receptors were not observed in the components forming the remaining strata of the gut wall. Flow cytometry and indirect immunofluorescence assays suggested that interaction of STa with its receptor on enterocytes and BBMVs prepared from the ileum was stronger than its interaction with the same preparations from other segments of the intestinal tract. Two different populations of enterocytes that differ in their forward scatter characteristics were observed upon analysis by flow cytometry (Fig. 2A). Forward scatter analysis suggested the existence of populations of enterocytes that are of two different sizes. Enterocytes that have large forward scatter values (large enterocytes) represented only 10% of the total population of enterocytes tested by flow cytometry. Flow cytometry analysis of the different sized enterocyte populations revealed similar trends in the fluorescent profiles on enterocytes prepared from different segments of newborn calf intestine. Only a single population of cells was observed upon flow cytometric analysis of enterocytes prepared from the small intestine of adult cattle (Al-Majali, unpublished observations). During the suckling period, the mucosa throughout the intestinal tract is characterized by long villus structures. Small enterocytes start migrating form the crypts to the tip of the villus. In the middle of the villus, enterocytes became larger but start getting smaller as they continue migrating to the tip of the villus [31]. This turnover process of the intestinal mucosa may explain the result of the forward scatter analysis of the enterocytes obtained from newborn calves. These enterocytes have a high percentage of cells with high forward scatter values when compared to enterocytes from adult animals.

The significant increase in the fluorescence intensities on enterocytes and BBMVs prepared from the ileum can be attributed either to an increase in either STa receptor density or in the affinity of this receptor to STa toxin. To

investigate this issue, [^{125}I]STa binding assay was performed on enterocytes and BBMVs prepared from the different segments of newborn calves.

The [^{125}I]STa binding data suggested that significantly higher numbers of STa receptors were present on enterocytes and BBMVs prepared from the ileum than on enterocytes from other intestinal segments. Previous reports in humans, avian and rats [18,32–34] suggested that the number of STa receptors decreases on intestinal enterocytes prepared from the distal parts of the small intestine. Data from this study suggest an increase in the affinity of the receptors to STa and an increase in their density on enterocytes as the distal end of the small intestine of newborn calves is approached. This is the first report that describes the STa receptor affinity and density along the intestinal tract of newborn calves. Previous studies have suggested that STa receptor density changes along the vertical axis of most mammals' small intestines (villus/crypt unit). They found that the STa receptor number is greatest in enterocytes forming the proximal region of the villus along the intestinal tract [18,32,35]. A similar gradient of STa receptor mRNA levels along the villus/crypt axis has been demonstrated in the rat small intestine [36]. This phenomenon can be attributed to the active cell division, which occurs primarily in the intestinal glands (crypts) [37,38]. Enterocytes that migrate to the tip of the villus possess a lower number of STa receptors and primarily perform absorptive functions [18,37,38].

In summary, this study described, for the first time, the distribution of STa receptors throughout the intestinal tract of newborn calves. We found that both the number of receptors and their affinity to STa were higher on ileal epithelium than other intestinal segments of the newborn calves. This study suggests that, in newborn calves, the ileum is the major part of the intestinal tract that is affected in the course of secretory diarrhea caused by STa-producing *E. coli* strains.

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