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Use of flow cytometry to measure the interaction between *Escherichia coli* heat-stable enterotoxin and its intestinal receptor in mice

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Abstract

Binding of *Escherichia coli* heat-stable enterotoxin (STa) to its putative receptor on the brush border membrane of enterocytes is a prerequisite for the induction of diarrhea in infected humans and animals. Humans and animals of different ages vary in their susceptibility to the effect STa, perhaps due to the difference in STa interaction with its intestinal receptor. Flow cytometry was compared to indirect immunofluorescence and ^{125}I–STa binding assays to measure the STa–enterocytes receptor interaction in different age groups of Swiss Webster mice (2-, 7-, 14-day-old). Flow cytometry indicated stronger interaction between STa and its putative receptor on enterocytes from the 2-day-old mice than enterocytes from older mice. ^{125}I–STa-binding assay suggested that the stronger fluorescence intensity on enterocytes from younger mice is due to higher STa receptor density and higher receptor affinity to STa. Flow cytometry is more sensitive quantitative assay to measure the interaction between STa and its intestinal receptor than indirect immunofluorescence microscopy.

Keywords: Flow cytometry; Heat-stable enterotoxin; Receptor; *Escherichia coli*

1. Introduction

Virulence factors that enable enterotoxigenic *Escherichia coli* (ETEC) strains to cause diarrheal disease during the first weeks of life include specific surface fimbriae, which mediate bacterial adherence to brush border membranes, and enterotoxins that stimulate intestinal secretion (Cohen et al., 1988; Kubota et al., 1989). ETEC produce two types of heat-stable enterotoxins, STa and STb (Saeed et al.,

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; STa, heat-stable enterotoxin; HPLC, high performance liquid chromatography; SMA, suckling mouse assay; BSA, bovine serum albumin; DTT, dithiothreitol

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1986; Sears and Kaper, 1996). STa is a low-molecular-weight nonantigenic protein that induces secretory response in suckling mice, rats, rabbits, calves and piglets (Dreyfus et al., 1984; Saeed et al., 1986). STa has been observed to alter dramatically guanosine 3′,5′-cyclic monophosphate (cGMP) metabolism via the activation of intestinal guanylate cyclase followed by a blockade of inward ion transport and subsequent secretion of water into the intestinal lumen (Gasstra and Graaf, 1982; Sack, 1990; Osek and Truszczynski, 1992). STa/receptor binding has been studied in human, pig, and rat intestine. In all of these species, an increase in brush border membrane–STa receptor density was observed in the immature intestine (Cohen et al., 1986; Jaso-Friedmann et al., 1992). This coincides with the period of increased susceptibility to STa-induced diarrheal disease that occurs in early life of humans and animals.

Stevens et al. (1971) described two periods of increased porcine responsiveness to STa; during the first week of life and directly after weaning. It is not clear whether the susceptibility to ETEC–STa changes with age and if this change results from alterations in the density and/or affinity of the enterocytes receptors that are specific for this enterotoxin. The development of age-dependent resistance against ETEC diarrheal diseases was observed in more than one species of animals. In vivo intestinal secretion studies revealed that immature rat jejunum is much more sensitive to the secretory effect of STa than adult jejunum (Cohen et al., 1986). More receptors were present in the jejunum membranes from the younger animals than from the adult animals, although STa-induced stimulation of guanylate cyclase increased with increasing age (Cohen et al., 1986; Guarino et al., 1987).

Competitive binding assay using radiolabeled STa (^{125}I–STa), enzyme-linked immunosorbent assay, and indirect immunofluorescence assay were used to study the STa/receptor interaction (Lockwood and Robertson, 1984; Cohen et al., 1986; Saeed et al., 1986; Jaso-Friedmann et al., 1992). In this study, we describe the use of flow cytometry as a simple, reliable quantitative assay to measure the interaction between STa and its enterocytes associated receptor. Flow cytometry was compared with indirect immunofluorescence and ^{125}I–STa binding assays to evaluate its utility in measuring the STa/receptor on enterocytes isolated from different age groups of mice.

2. Materials and methods

2.1. STa purification

ETEC isolated from calves with clinical diarrhea were grown in a 15 L asparagine-salts medium as described previously (Staples et al., 1980). STa was purified as described by Saeed and Greenberg (1985). Briefly, Bacterial cell-free harvest was obtained by tangential flow filtration through 0.2-μm cassette in a Millipore Pellicon system (Millipore, Bedford, MA). Filtrates was desalted and concentrated by Amberlite XAD-2 chromatography. Reverse-phase batch adsorption chromatography using Bondesil (Analyticum International, Harber City, CA) was performed on the Amberlites XAD-2 fractions. STa was further purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Voydac RP-300 C-8 column (Vydac, Hesperia, CA) using Shimadzu LC-600 pumps and SPD-10AV UV-detector (Shimadzu, Columbia, MD). STa was eluted by applying a gradient of a 0 to 80% HPLC-grade methanol in 0.1% TFA. STa activity was determined by suckling mouse assay (SMA) (Giannella, 1976) and total protein was estimated by the modified method of Lowry (Peterson, 1977).

2.2. Conjugation of STa to bovine serum albumin (BSA)

Due to the fact that STa is a small molecule and is non-immunogenic, it was conjugated to BSA as a carrier through its amino terminus to preserve its biological activity that is associated with the carboxyl terminus of the toxin molecule. To promote the conjugation of STa molecule to BSA through its amino terminus, BSA was succinylated as described previously (Habeeb, 1967). STa was conjugated to succinylated BSA in a reaction mixture containing 35 mg of succinylated-BSA and 5 mg of STa. Sixty milligrams of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was added and pH was adjusted to 9. After overnight incubation at 4°C, the mixture was dialyzed against 4 L of

distilled water. The biological activity of STa–BSA was evaluated by SMA.

2.3. Preparation of polyclonal STa-specific antiserum

Six 2 kg female New Zealand albino rabbits were immunized by injection at multiple intradermal sites with STa–BSA cinjugates [1 ml of 1 mg/ml in phosphate buffered saline (PBS) mixed with 1 ml of complete Freund's adjuvant]. Each rabbit was then boosted 1 month later by similar intradermal inoculations of 1 ml of STa–BSA conjugate (1 mg/ml PBS) mixed with 1 ml of incomplete Freund's adjuvant six boosters were given at monthly intervals. Blood samples were taken from rabbits 3 weeks after the booster immunization. Sera were collected and titrated for their STa-neutralization using SMA.

2.4. Experimental animals

Three different age groups (2-, 7-, 14-day-old) of Swiss Webster mice were used in this experiment (8–10 mice in each group). Mice were euthanized by ether anesthesia followed by cervical dislocation, and single cell suspensions of enterocytes were prepared from each group as described below.

2.5. Preparation of suckling mouse enterocytes

Suckling mouse enterocytes were prepared as described previously (Al-Majali et al., 1998). Briefly, parts from the small intestine were chopped into small pieces and placed in a solution contains dithiothreitol (DDT)–EDTA solution (1.5 mM EDTA; 0.5 mM DTT, and 1000 IU of penicillin/streptomycin dissolved in phosphate buffer solution PBS (pH 7.2)). After 45 min incubation at room temperature, the solution was filtered through a cotton filter to remove particulate material. Enterocyte suspension was filtered through a nylon-mesh filter (50 μm), centrifuged at $1000 \times g$ for 5 min and then washed three times with PBS to remove any traces of DTT. The population of cells harvested was monitored by periodic wet mount examination through the whole procedure to assess the quantity and quality of the isolated enterocytes. Only cell suspensions with $> 80\%$ viable cells were used for flow cytometry, indirect immunofluorescence and ^{125}I–STa binding assay.

2.6. Flow cytometry analysis

Enterocytes were prepared for staining by three additional washes with PBS, pH 7.2, containing 0.5% BSA. In a volume of 100 μl, 10^5 enterocytes in PBS–BSA were incubated with 50 μl of HPLC purified STa (10 μg/ml of 10 mM PBS) for 45 min at 37°C. After washing three times in PBS–BSA, enterocytes were resuspended in 100 μl of PBS–BSA. Fifty microliters of STa-specific antiserum produced in rabbits was diluted 1:10 in PBS, added to the enterocyte suspension and incubated for 30 min at 4°C. Cells were washed three times with PBS–BSA and resuspended in 100 μl of PBS–BSA. Fifty microliters of goat anti-rabbit-IgG–FITC-conjugated antibody (KPL, Gaithersburg, MD) diluted 1:100 in PBS was added to the enterocyte suspension and incubated for 30 min on ice. Cells were washed three times with PBS–BSA, resuspended in 1.0 ml of PBS, and kept on ice until flow cytometric analysis was performed. As a negative control, similar samples were incubated only with the secondary FITC-conjugated antibody and used to determine the threshold of specific staining. Flow cytometric analysis was performed using the Epics ELITE flow cytometer (Coulter Electronics, Hileah, FL). FITC-stained cells were excited by using 15 mW of 488 nm argon laser light. Calibration beads were run and the mean fluorescent intensity was set at a fixed value, which was maintained throughout the experiment.

2.7. Indirect immunofluorescence assay

Enterocytes smears were incubated with 50 μl (100 μg/ml of 10 mM PBS) of HPLC-purified STa for 45 min at 37°C. After washing three times with PBS (pH 7.4), slides were incubated at 37°C for 45 min with 50 μl of 1:10 diluted anti-STa antibody produced in rabbit. Slides were washed three times in PBS and reincubated with 50 μl of 1:100 diluted anti-rabbit-IgG–FITC-conjugated antibody (KPL). After a 45-min incubation, slides were rinsed in PBS and examined using a Nikon labophot epi-fluorescence microscope.

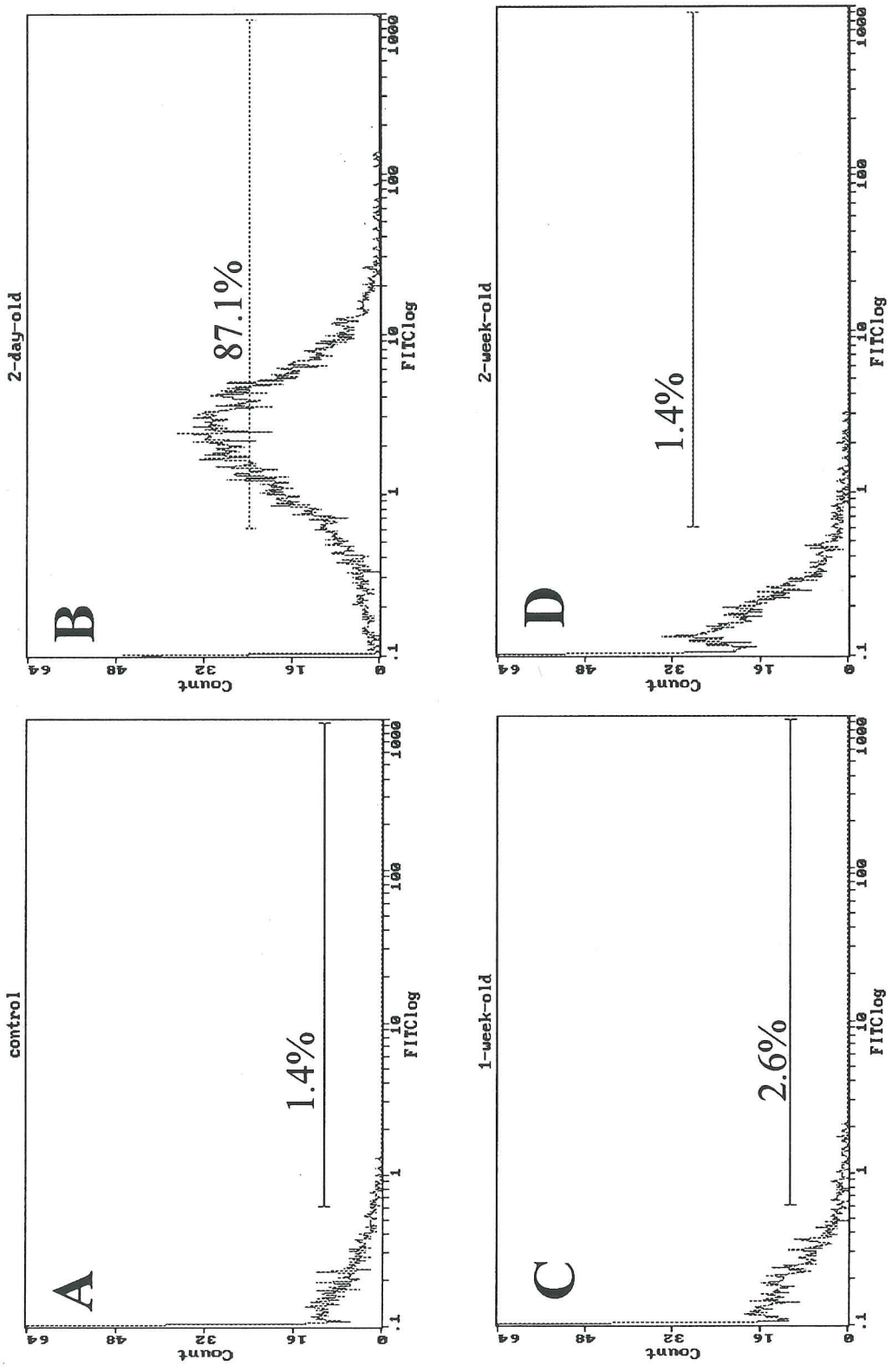

Fig. 1. Representative flow cytometric histograms for the STa–enterocytes interaction in different age groups of mice. (A) Control, no STa toxin was added; (B) enterocytes from 2-day-old mice; (C) enterocytes from 7-day-old mice; (D) 14-day-old mice. Enterocytes where incubated with STa, rabbit anti-STa antibodies, and stained with anti-rabbit-IgG–FITC conjugated antibodies. Those trends were consistent upon repeating the experiment five times. Fluorescence of signal-positive enterocytes is expressed in percent in each histogram.

2.8. STa iodination

HPLC-purified STa was radioiodinated in a reaction mixture that contained the following: STa, 100 μg; 0.2 M sodium phosphate (pH 7.2), 45 μl; one Iodo-bead® (Pierce, Rockford, IL); Na–^{125}I (NEN, Boston, MA), 1.0 mCi; and 2% D-glucose, 25 μl. After 15 min incubation at room temperature, radiolabeled STa (^{125}I–STa) was separated from free iodine using a Sep-Pack C-18 cartridge column (Waters Associates, Milford, MA). The column was prewashed with 10 ml 100% methanol and equilibrated with 10 ml distilled water. Stepwise elution of the ^{125}I–STa was performed with (i) 10 ml of 30% methanol (HPLC-grade) in 0.1% trifluoroacetic acid (TFA), (ii) 10 ml of 60% methanol in 0.1% TFA, (iii) 10 ml of 100% methanol in 0.1% TFA.

2.9. Binding assay

Reaction mixtures containing isolated mice enterocytes (2×10^3), PBS–BSA and ^{125}I–STa (20–640 nM) were incubated in a final volume of 200 μl for 40 min at 37°C in a shaking water bath. Unbound ^{125}I–STa was removed from bound ^{125}I–STa by vacuum filtration (Millipore), using 1-μm, 2.5 cm GF/B glass filters (Whatman, Maidstone, England). Total binding was measured in a reaction mixture that did not contain the unlabeled STa, whereas nonspecific 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. All experimental points were determined in duplicate. Specific binding data were used to calculate the apparent dissociation constants (K_d) and the maximum number of STa receptors (B_{max}) associated with enterocytes (Scatchard, 1949).

3. Results

3.1. STa purification and STa–BSA antibodies production

STa was isolated and purified to homogeneity using the protocol described in Section 2. Amino acid composition and sequence analysis of the purified STa revealed homology with STa purified from human, porcine, and bovine origin (Lazure et al., 1982; Saeed et al., 1983; Thompson and Giannella, 1985). Only two of the six immunized rabbits produced sera of high STa antibody titers. However, the STa-neutralization titer which was determined using the SMA indicated that the pooled serum from the two rabbits had an STa-neutralization titer of 1:10,000. This titer is higher than similar reagents reported previously (Lockwood and Robertson, 1984).

3.2. Flow cytometry analysis

A histogram showing significantly increased fluorescence intensity was associated with the 2-day-old suckling mice enterocytes that were stained with rabbit anti-STa and anti-rabbit-IgG–FITC-con-

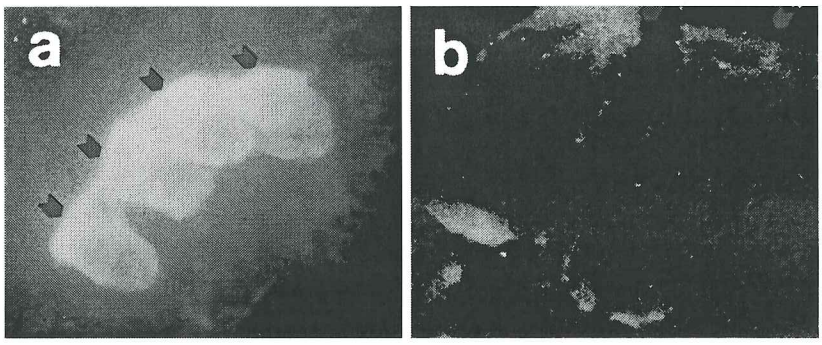

Fig. 2. Immunofluorescence staining of STa interaction with mice enterocytes. (a) 2-day-old suckling mouse enterocytes incubated with STa and anti-STa–rabbit IgG antibodies and stained with FITC-conjugated anti-rabbit IgG antibodies; (b) control, 2-day-old suckling mouse enterocytes incubated only with STa and stained by FITC-conjugated anti-rabbit IgG antibodies (×1000).

Table 1
Binding properties of ^{125}I–STa to enterocytes from mice of different age groups[a]

Mouse age group	Specific binding (%)[b]	Dissociation constant (nM)	STa receptor density (nM/mg protein)
2-day-old	94	75	7.2
7-day-old	72	125	0.30
14-day-old	62	1430	0.36

[a] Similar trends were obtained upon repeating the experiment.
[b] Each number represents the average of six readings.

jugated antibodies. Only weak fluorescence was demonstrated from similarly prepared and processed samples from the 7- and 14-day-old mice. No fluorescence was observed on processed enterocytes from any age groups when no STa was added. The staining results of freshly isolated enterocytes from different age groups of suckling mice are shown in Fig. 1.

3.3. Indirect immunofluorescence assay

Indirect immunofluorescence study of enterocytes from STa-susceptible mice revealed the localization of intensely stained areas mostly at the brush border membrane region. Enterocytes obtained from small intestine of 2-day-old suckling mice showed an intensely fluorescent brush border membrane after treatment with rabbit anti-STa and anti-rabbit-IgG–FITC-conjugated antibodies (Fig. 2). Fluorescence intensities were relatively low in the intestinal sections and enterocytes smears obtained from 7- and 14-day-old mice when compared with that of the 2-day-old suckling mice.

3.4. Stoichiometry of STa receptor

The binding of ^{125}I–STa to enterocytes from each group of mice was saturable, and reached a plateau. The specific binding of ^{125}I–STa to enterocytes from 2-day-old mice was about 2-fold higher than the specific binding of ^{125}I–STa to enterocytes obtained from 7- and 14-day-old mice (Table 1). Scatchard analysis of specific binding data suggests the existence of a single class of STa receptors associated with enterocytes from different age groups. Calculation of K_d and B_{max} suggested higher affinity and receptor density for STa in the 2-day-old suckling mice enterocytes than in other age groups. STa receptor density of the 2-day-old suckling mice was 20-fold higher than that of the 7- and 14-day-old mice (Table 1). The dissociation constant of STa receptor of enterocytes obtained from 2-day-old suckling mice (75 nM) was 10-fold lower than that obtained from 14-day-old mice.

4. Discussion

The age-dependent resistance to diarrheal disease caused by ETEC was first reported in pigs. Moon and Whipp (1970), found that some strains of ETEC cause secretory diarrhea only in neonatal pigs under 2 weeks old, whereas, other strains have the ability to cause diarrhea in neonatal and older pigs. Enterotoxins are the most important virulence factor of ETEC and are considered the immediate mediator of diarrhea (Rao et al., 1981; Osek and Truszczynski, 1992). High doses of STa were found to affect the secretory response in ligated intestinal loops, and addition of STb to STa-treated loops increased this secretory response. Although differences may exist in the sensitivity of neonatal and adult hosts to bacterial enterotoxins, little is known about changes of enterotoxin receptor affinity and density in the first weeks after birth.

In this study, interaction of STa with its putative receptor on enterocytes obtained from mice of different age groups was demonstrated using flow cytometry. The absence of fluorescence in the control group, where no toxin was added, suggested a specific interaction between STa and its putative receptor. The significant increase in the fluorescence intensity

in the 2-day-old suckling mice enterocytes (Fig. 1), which were treated with STa, rabbit anti-STa and anti-rabbit-IgG–FITC conjugated antibodies, may be due to an increase in either the receptor number or the affinity of STa to these receptors. To investigate whether the high fluorescence intensity obtained from the flow cytometry is due to changes in the receptor affinity or density, ^{125}I–STa binding assay was performed.

The ^{125}I–STa binding affinity data suggested that, significantly higher number of STa receptors was present on the enterocytes of the 2-day-old mouse group than older mice. The number of the STa receptors on the enterocytes of the 7- and 14-day-old mice was significantly lower than that of the 2-day-old mice (Table 1). Unlike previous reports in pigs (Jaso-Friedmann et al., 1992), our data suggest an increase in the STa receptor affinity in the 2-day-old suckling mice (Table 1). This age-dependent affinity of STa receptors may be due to conformational or structural changes in the extracellular domain of the guanylate cyclase protein. Further investigation is needed to elucidate this age-dependent affinity.

Binding of ^{125}I–STa to mice intestinal cells was shown to be rapid, specific, saturable, temperature dependent and belongs to a single class of receptors. It is unclear why STa receptors exist in a larger number on enterocytes from neonatal animal. It is possible that the STa receptor functions as a receptor for growth promoting peptide(s) and that an increased number of receptors for this peptide would be needed in the intestine of the neonate. It is noteworthy that, recently, we studied the effect of dietary insulin on the response of suckling mice enterocytes to STa. Insulin was found to up-regulate this response (Al-Majali et al., 1998).

It is likely that multiple factors contribute to the increased susceptibility of newborn animals to ETEC. This predilection might be variably expressed on the basis of permissive host factors, including brush border membrane changes that may be induced by dietary antigens, stress, or by environmental factors.

This report describes the use of flow cytometry to study the interaction of STa with its putative receptor. Flow cytometry analysis has the benefit of analyzing individual cells and providing multiparametric data acquisition, high-speed analysis and effective cell sorting (Davey and Kell, 1996). A particularly powerful feature of flow cytometric procedures is that they permit analysis of the covariance of different determinants (Kell and Sonnleitner, 1995; Davey and Kell, 1996).

Conventional fluorescent microscopy analysis results only in inaccurate estimates of fluorescence intensity due to the scoring protocol used (+ + + +, for high fluorescent intensity; +, for relatively low fluorescent intensity). Using flow cytometry, studying the STa/receptor interaction was possible due to the accurate determination of the intensity of fluorescence on enterocytes from different age groups of suckling mice.

In summary, we have demonstrated the utility of flow cytometry in the study of the interaction of STa with its enterocytes receptor in mice. Supported by indirect immunofluorescence and ^{125}I–STa binding assays, results of this study suggest that the numbers and affinity of the STa receptor in mice are age-dependent. These results may further explain the increased susceptibility of immature and young animals to STa-mediated diarrheal disease. This model will be utilized in future studies for further investigations of the mechanism of STa-mediated diarrheal disease in humans and animals.

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