Characterization of the Interaction of *Escherichia coli* Heat-Stable Enterotoxin (STa) with its Intestinal Putative Receptor in Various Age Groups of Mice, Using Flow Cytometry and Binding Assays

Ahmad M. Al-Majali,[1] J. Paul Robinson,[2] Elikplimi K. Asem,[2] Carlton Lamar,[2] M. James Freeman,[1] and A. Mahdi Saeed[1]*

Background and Purpose: Enterotoxigenic *Escherichia coli* heat-stable enterotoxin (STa) is a major cause of diarrhea in young animals. Age-dependent variation in the density and affinity of the mouse enterocyte receptors specific for STa was investigated.

Methods: Four age groups (2-day-, 1- and 2-week-, and 2-month-old) of Swiss Webster mice were studied (8 to 10 mice/group). Flow cytometry and radiolabeled STa (^{125}I-STa) assays were used as reliable quantitative measures for characterization of STa-enterocyte receptor interaction.

Results and Conclusions: Interaction of STa with its putative receptor was stronger for enterocytes of 2-day-old mice. Scatchard analysis of ^{125}I-STa-receptor interaction suggested that STa-receptors exist at higher numbers on enterocytes from 2-day-old (7.2 nmol/mg) than older (0.30, 0.36, and 0.40 nmol/mg for 1-week-, 2-week-, and 2-month-old mice, respectively). Additionally, receptors from 2-day-old mice had greater affinity for STa (K_d = 75 nM) than did receptors from older mice (K_d = 125, 1,430, and 1,111 nM for 1-week-, 2-week-, and 2-month-old mice, respectively). Density of STa receptors on enterocytes and their affinity to STa may determine extent of binding and severity of the secretory response, and may explain the high susceptibility of newborn animals and human infants to STa-mediated diarrhea.

Secretory diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is a major cause of death among human infants and young animals in developing countries (1–4). Virulence factors that enable ETEC strains to cause diarrheal disease during the first days of life include specific surface fimbriae, which mediate bacterial adherence to intestinal epithelial cells, and enterotoxins that stimulate intestinal secretion (5–7). The ETEC produce various types of enterotoxins: heat-labile enterotoxin (LT), and two types of heat-stable enterotoxins (STa and STb) (8, 9).

Heat stable toxin-mediated diarrhea is more common and more severe in young animals and human infants (2, 10–12). The STa is a cysteine-rich, poorly immunogenic, 18- or 19-amino acid peptide with molecular mass of 2 kDa (5, 9). The toxin has been observed to markedly alter guanosine 3',5'-cyclic monophosphate (cGMP) metabolism via activation of intestinal guanylate cyclase followed by blockade of inward ion transport and subsequent secretion of fluids into the intestinal lumen (13, 14). The sequence of events that ends in stimulation of intestinal fluid secretion and diarrhea is initi-

ated by STa binding to a specific receptor located on the brush border membrane of the intestinal epithelial cells of the host (6, 15). The events end with an increase in intracellular cGMP causing intraluminal accumulation of fluids and electrolytes, which leads to diarrhea (13, 14, 16, 17). Some reports suggest that the STa-receptor has a polymeric structure of a glycoprotein with molecular mass of 140 kDa and a topologic organization that is similar to the atrial and brain natriuretic peptide receptors (18, 19). The STa-receptor is believed to be part of the extracellular motif of the brush border-associated guanylyl cyclase (3, 18, 20).

The STa-receptor binding has been studied in human, pig, and rat intestine. In all these species, a high density in brush border membrane STa-receptor density was observed in the intestine of immature animals (6, 10, 11). 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. (21) described two periods of increased pig 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 whether this change results from alterations in the density and/or affinity of the enterocyte's receptors that are specific for this enterotoxin. Development of age-dependent resistance

Departments of Veterinary Pathobiology[1] and Basic Medical Sciences,[2] School of Veterinary Medicine, Purdue University, West Lafayette, Indiana
*Address correspondence to: Dr. A. Mahdi Saeed, Department of Veterinary Medicine, Purdue University, West Lafayette, IN 47907-1243.

against ETEC diarrheal diseases was observed in more than one species of animals. Whipp et al. (22) reported a difference between the response of 7-day- and 7-week-old pigs to STa and STb. Culture supernatant containing STa induced secretory response only in the 7-day-old pigs, whereas culture supernatant containing STa and STb induced secretory response in both ages (22). Previous studies on the effect of age on the interaction between STa and its putative receptor indicated that immature rat jejunum was much more sensitive to the secretory effect of STa than was adult jejunum (10, 15).

In this study, we hypothesized that the susceptibility of the 2-day-old mice that are used in the suckling mouse assay of ETEC-STa is modulated by a high number of STa-receptor on their enterocytes, and that the affinity of these receptors to the STa toxin may be age-dependent. Currently, the suckling mouse model is the only reliable bioassay for ETEC-STa. Flow cytometry, ^{125}I-STa affinity binding, and indirect immunofluorescence assays were used to characterize the interaction of ETEC-STa with its putative receptor on enterocytes of various age groups of mice.

Materials and Methods

Purification of E. coli heat-stable enterotoxin (STa): The STa was produced and purified to homogeneity, using the methods described by Staples et al. (23) and modified by Saeed and Greenberg (8).

Experimental animals: Four age groups (2-day, 1-week and 2-week, and 2-month-old) of Swiss Webster mice, of both sexes, were studied. Eight to ten animals were used in each group to produce sufficient numbers of enterocytes for the binding procedures. Mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). Vendor surveillance indicated that mice were free of all known bacterial, viral, and parasitic pathogens. Mice were housed in polycarbonate open (nonfiltered) cages containing Care-Fresh animal bedding (Absorption Corp., Bellingham, Wash.) and were fed Autoclavable Laboratory Diet 5001 (Purina Mills, Inc., Richland, Ind.) and tap water. The mouse room had 13 to 15 complete air changes/h and was maintained at 22 ± 1°C temperature with 45 ± 2% relative humidity and a 12/12-h light/dark cycle. All animal procedures were approved by the Purdue University Animal Care and Use Committee, in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Mice were euthanized by ether anesthesia followed by cervical dislocation, and single cell suspensions of enterocytes were prepared from each group.

Isolation of suckling mouse enterocytes: The entire intestine from each group was collected and placed in tissue culture medium (Medium 199). The intestine was moved to a petri dish containing dithiothreitol (DTT)-EDTA solution (1.5 mM EDTA; 0.5 mM dithiothreitol and 10,000 IU of penicillin/10 mg of streptomycin dissolved in phosphate-buffered saline solution [PBSS] [pH 7.2]). The intestine was chopped into small pieces, using a sterile blade, and was left in the DTT solution for 45 min, then was passed through a cotton filter to remove particulate material. Enterocyte suspension was passed through a nylon-mesh filter (50 μm), centrifuged at 1,000 X g for 5 min, then washed three times with PBSS

to remove any traces of DTT. The population of cells harvested was monitored by periodic wet mount examination throughout the entire procedure to assess the quantity and quality of the isolated enterocytes. The small size of the intestine of the 2-day-old suckling mice precluded us from obtaining a sufficient number of enterocytes from each animal separately; therefore, enterocytes from each group were pooled together. Cell counts and cell viability were determined by exclusion of 0.2% trypan blue-stained cells. Only cell suspensions that contained >80% viable cells were used for indirect immunofluorescence, flow cytometric analysis, and ^{125}I-STa binding assay.

Indirect immunofluorescence assay: Intestinal cryostat-cut sections were incubated with 50 μl (100 μg/ml of 10 mM PBSS) of high-performance liquid chromatography (HPLC)-purified STa for 45 min at 37°C. After being washed three times with PBSS (pH 7.4), slides were incubated at 37°C for 45 min with 50 μl of 1:10 diluted anti-STa antibody produced in rabbits. Slides were again washed three times in PBSS and reincubated with 50 μl of 1:100 diluted anti-rabbit IgG/FITC-conjugated antibody (KPL, Gaithersburg, Md.). After a 45-min incubation, slides were rinsed in PBSS and examined using a Nikon labophot epi-fluorescence microscope.

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

Iodination of STa: The 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, Ill.); Na^{125}I (New England Nuclear, Boston, Mass.), 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, Mass.). The column was prewashed with 10 ml of 100% methanol and was equilibrated with 10 ml of distilled water.
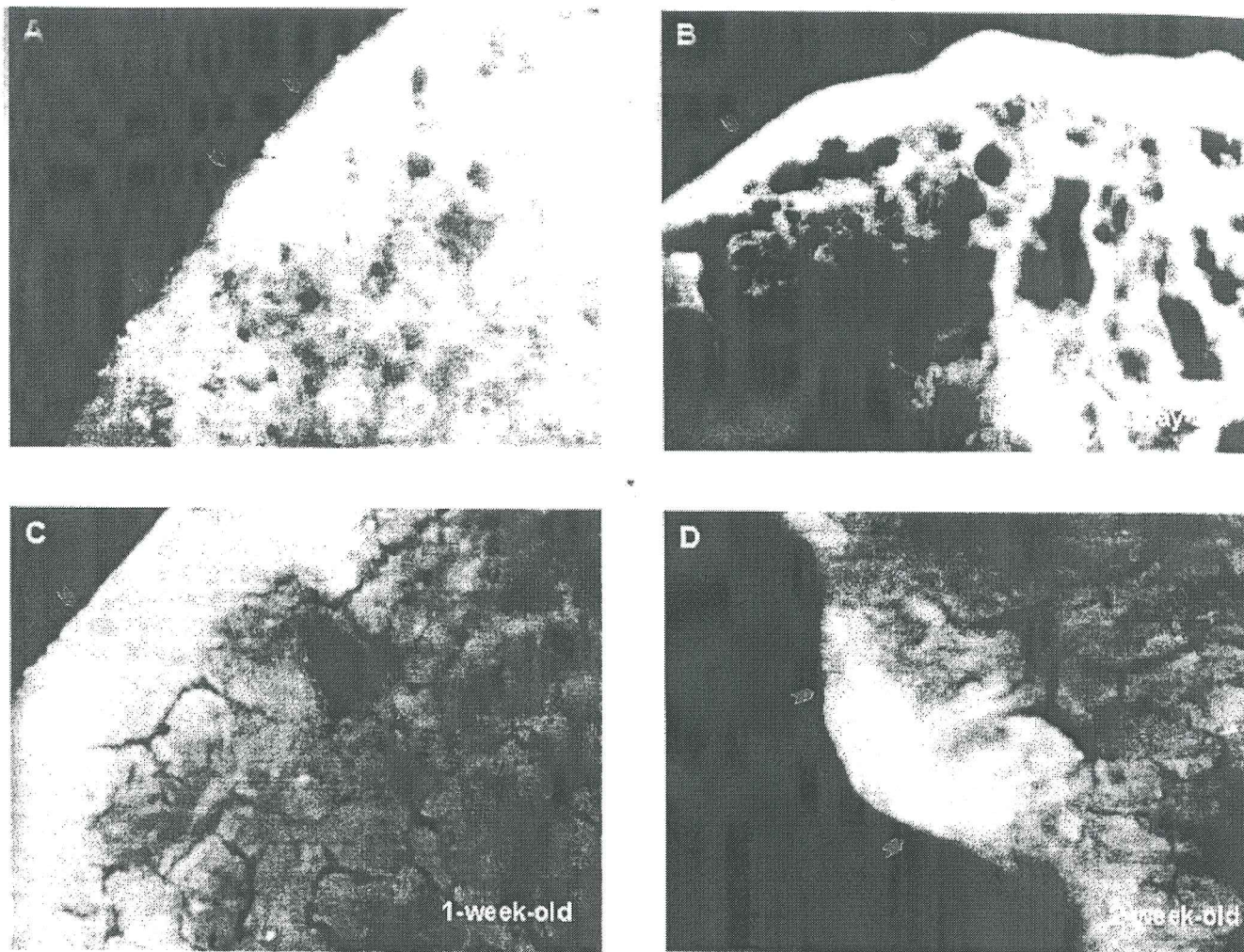
Figure 1. Immunofluorescent staining of *Escherichia coli* heat-stable (STa) toxin in cryostat-cut sections of small intestine from mice. (A) Control, 2-day-old suckling mouse intestinal section incubated only with STa and stained by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibodies. (B) Two-day-old suckling mouse intestinal section incubated with STa and rabbit origin anti-STa IgG antibodies and stained with FITC-conjugated anti-rabbit IgG antibodies. (C and D) Intestinal sections from 1- and 2-week-old mice incubated with the same reagents as in B. Magnification, x1,000.

Stepwise elution of the ^{125}I-STa was performed with 10 ml of 30% methanol (HPLC grade) in 0.1% trifluoroacetic acid (TFA); 10 ml of 60% methanol in 0.1% TFA; and 10 ml of 100% methanol in 0.1% TFA.

Protein concentration was measured by use of the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.), using BSA as the standard. The purified radiolabeled STa had a 350 mCi/nmol specific activity and retained its secretory activity in the 2-day-old suckling mice.

Binding assay: Reaction mixtures containing isolated mouse enterocytes (2×10^3), PBSS-BSA, and ^{125}I-STa (20 to 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 Corp., Bedford, Mass.), 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 a 1,000-fold excess of unlabeled STa. Specific binding was cal-

culated by subtracting nonspecific binding from the total binding. A Scatchard plot for the ^{125}I-STa specific binding data was 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 maximal number of STa receptors (B_{max}) were calculated, using the Rosenthal-Scatchard equation (24, 25).

Results

Indirect immunofluorescent assay: Indirect immunofluorescent assay of intestinal sections from STa-susceptible mice revealed the localization of intensely stained areas mostly at the brush border membrane region. Cryostat-cut sections obtained from the small intestine of 2-day-old suckling mice had an intensely fluorescent brush border membrane after treatment with rabbit anti-STa and anti-rabbit IgG/FITC-conjugated antibodies (Figure 1). Some fluorescence was found in some focal areas inside the mucosa of the intestine. Fluorescence intensities were low in the intestinal sections and enterocytes smears obtained from 1- and 2-
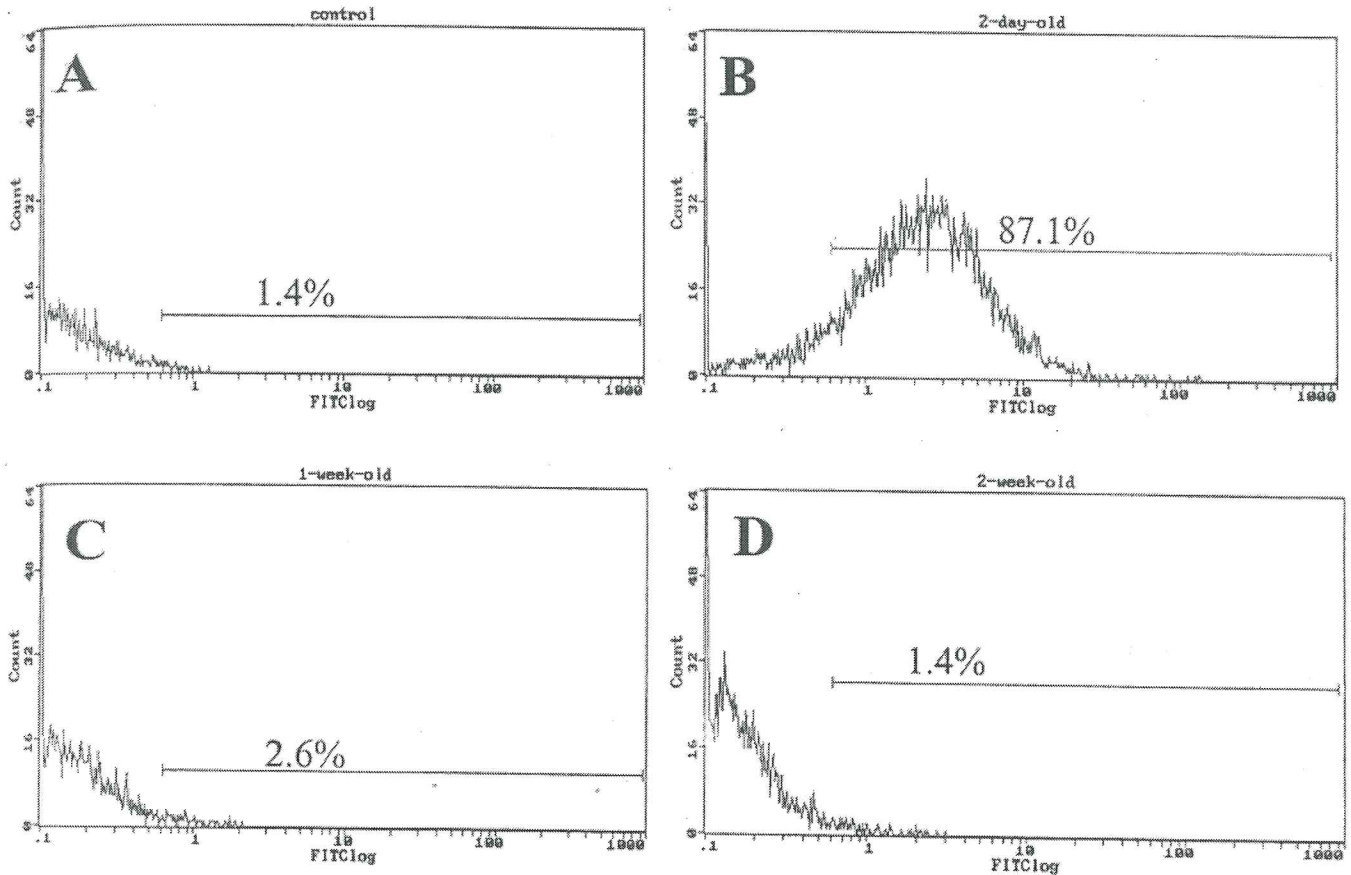
Figure 2. Representative flow cytometric histograms from various age groups of mice. (A) control, STa toxin was not added. (B) Enterocytes from 2-day-old mice. (C) Enterocytes from 1-week-old mice. (D) Enterocytes from 2-week-old mice. Enterocytes were incubated with STa and rabbit anti-STa IgG antibodies, and stained with anti-rabbit IgG/FITC-conjugated antibodies. The values 1.4, 87.1, 2.6, and 1.4% in A, B, C, and D, respectively, represent the percentage of fluorescence associated with enterocytes in the various age groups of mice.

week-old mice, compared with those of the 2-day-old suckling mice, suggesting that fluorescence intensity is inversely related to age.

Flow cytometric analysis: The binding of STa to its putative receptor was studied, using flow cytometric analysis. A histogram showing significantly increased fluorescence intensity was associated with the 2-day-old suckling mouse enterocytes that were stained with rabbit anti-STa and anti-rabbit IgG/FITC-conjugated antibodies (Figure 2). Only weak fluorescence was detected in similarly prepared and processed samples from the 1- and 2-week-old mice. Fluorescence was not observed on processed enterocytes from any age groups when STa was not added.

Effect of ^{125}I-STa concentration on binding: Binding assays with enterocytes from each age group were performed to characterize the association of STa with its putative receptor on the enterocyte surface. The binding of ^{125}I-STa to enterocytes from each group of mice was saturable and reached a plateau (Figure 3). The specific binding of ^{125}I-STa to enterocytes from 2-day-old mice was about 1.3- to 1.57-fold higher than the specific binding of ^{125}I-STa to enterocytes obtained from 1-week-, 2-week-, and 2-month-old mice (Table 1). Nonspecific binding in the 2-day-old mouse enterocytes accounted for only 6% of the total binding, whereas nonspecific binding for the 1-week-, 2-week-

and 2-month-old mouse enterocytes accounted for 28 to 40% of the total binding (Table 1).

Stoichiometry of ^{125}I-STa binding to the various age groups of mouse enterocytes: Scatchard analysis of specific binding data suggested the existence of a single class of STa receptors associated with enterocytes from different age groups. Calculation of K_d and B_{ma} suggested higher affinity and receptor density for STa in the 2-day-old suckling mouse enterocytes than in those of other age groups. The STa-receptor density of the 2-day-old suckling mice was about 18- to 24-fold higher than that of the 1-week- and 2-week-, and 2-month-old mice (Table 1). The dissociation constant of STa-receptor of enterocytes obtained from 2-day-old suckling mice (75 nM) was about 14- to 19-fold lower than that obtained from 2-week- and 2-month-old mice. The K_d of the 1-week-old suckling mice (125 nM) was 1.7-fold higher than that of the 2-day-old suckling mice. The ^{125}I-STa binding properties are shown in Table 1.

Discussion

Age-dependent resistance to diarrheal disease caused by ETEC was first reported in pigs. Moon and Whipp (26) found that some strains of ETEC cause secretory diarrhea only in neonatal pigs <2 weeks old, whereas other strains have the ability to cause diarrhea in neonatal and older pigs. The
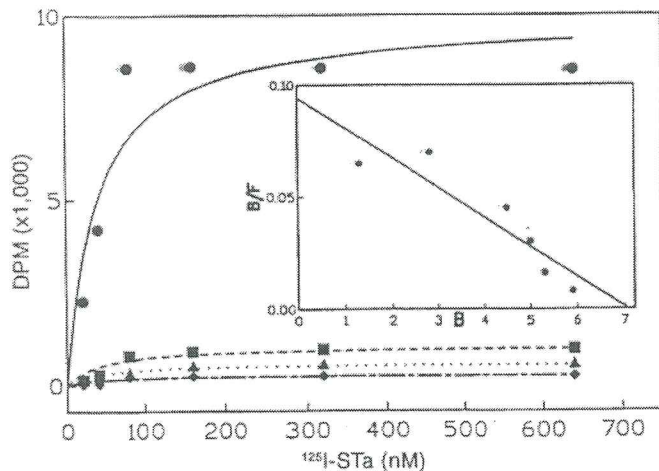
Figure 3. Specific binding of ^{125}I-labeled STa (^{125}I-STa) to enterocytes obtained from various age groups of mice in the presence of increasing concentrations of ^{125}I-STa. ●——●: enterocytes from 2-day-old suckling mice; ■---■, enterocytes from 1-week-old suckling mice; ▲—▲, enterocytes from 2-week-old mice; and ◆-·-◆, enterocytes from 2-month-old mice. Nonspecific binding was determined in incubation mixtures containing ^{125}I-STa and a 1,000-fold excess of unlabeled STa. Similar trends were obtained on repetition of the experiment. Inset represents Scatchard plot ⊛ = 0.93 for the 2-day-old mouse enterocytes. Scatchard plot analyses for the specific binding data of the various age groups are shown in Table 1.

Table 1. Binding properties of ^{125}I-labeled (^{125}I-STa) enterocytes from Swiss Webster mice of various ages

Mice[a] Age	Mice[a] Number	Specific binding (%)	Non-specific binding (%)	Dissociation constant (nM)	STa receptor density (nmol/mg of protein)
2 days	10	94 ± 3.00[b]	6 ± 2.00[b]	75 ± 2.00[b]	7.20 ± 0.67[a]
1 wk	10	72 ± 3.00	28 ± 2.00	125 ± 4.00	0.30 ± 0.10
2 wk	9	62 ± 1.00	38 ± 2.65	1,430 ± 18.93	0.36 ± 0.03
2 mo	8	60 ± 2.65	40 ± 3.46	1,111 ± 4.36	0.40 ± 0.07

[a]Enterocytes from each group were pooled together and were tested in triplicate.
[b]The differences between 2-day-old mice and 1-, 2-week-, and 2-month-old mice were significant ($P < 0.01$), using one-way ANOVA followed by pairwise comparison probabilities (Bonferroni correction).
Data are expressed as mean ± SD.

main virulence factors in *E. coli* that are associated with diarrhea in the first weeks of life include specific surface fimbriae that mediate bacterial adhesion as the initial step of colonization of the intestinal brush border membranes (7, 27), and enterotoxins that stimulate intestinal secretion (6–8, 11). Enterotoxins are considered the immediate mediator of diarrhea (3, 6, 8–11). Although differences may exist in the sensitivity of neonatal and adult hosts to bacterial enterotoxins, little is known about changes in enterotoxin receptor affinity and density in the first weeks after birth.

In this study, the presence of STa-receptors on enterocytes obtained from mice of various age groups was documented, using flow cytometric and indirect immunofluorescent assays. The absence of fluorescence in the control group, in which toxin was not added, suggested a specific interaction between STa and its putative receptor. The significant increase in the fluorescence intensity in the 2-day-old suckling mouse enterocytes, which were treated with STa and rabbit anti-STa and anti-rabbit IgG/FITC conjugated antibodies, must have been due to a higher density of the receptor or higher affinity of these receptors to STa (Figure 2). Similar results were obtained by use of indirect immunofluorescent examination (Figure 1). For further investigation of STa-receptor stoichiometry, ^{125}I-STa binding affinity to STa-receptors on mouse enterocytes was performed.

The ^{125}I-STa binding affinity data suggested that significantly higher numbers of STa-receptors were present on the enterocytes of the 2-day-old than older mice (Figure 3). The numbers of the STa-receptors on the enterocytes of the 1-week, 2-week-, and 2-month-old mice was significantly lower than that on enterocytes from 2-day-old mice (Table 1). Un-

like previous reports in pigs and rats (6, 10), our data suggest an increase in the STa-receptor affinity in the 2-day-old suckling mice (Table 1). This age-dependent affinity of the STa-receptor 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 2-day-old mouse enterocytes was specific in that the excess unlabeled STa reduced the amount of bound ^{125}I-STa by >90%. The amount of the nonspecific binding is probably due to hydrophobic interactions between the basolateral membranes of enterocytes and the ^{125}I-STa (28). Binding of ^{125}I-STa to mice intestinal cells was documented to be rapid, specific, saturable, and temperature-dependent (data not shown). Mouse enterocytes appear to have one class of STa-receptor, as suggested by the linear nature of the Scatchard plot curve ($r = 0.93$). It is unclear why STa-receptors exist in a larger number on enterocytes from neonatal animals. 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 insulin on the response of suckling mouse enterocytes to STa. Insulin was found to up-regulate this response (29).

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 environmental factors. The high susceptibility to ETEC might be augmented or more fully expressed in response to host or environmental factors.

We have described the use of flow cytometry to study the interaction of STa with its putative receptor. Use of conventional fluorescent microscopy 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, study of the STa/receptor interaction was possible through accurate determination of the intensity of fluorescence on enterocytes of the various age groups of mice.

In conclusion, we have documented, using flow cytometric, indirect immunofluorescent, and ^{125}I-STa binding assays, that in mice, STa-receptor numbers and affinity are age-dependent. The STa-receptor numbers and affinity were

higher in the 2-day-old suckling than older mice. Results generated from the mouse model may help to explain, in part, the increased susceptibility of immature and young animals to STa-mediated diarrheal disease. Further experiments are needed to determine the role of fimbriae in the age-dependent susceptibility of mice to the ETEC.

Acknowledgement

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