The Jejunal Secretory Response to *Escherichia coli* Heat-Stable Enterotoxin is Prolonged in Malnourished Rats

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ABSTRACT. Undernutrition in human infants is associated with more prolonged episodes of diarrheal disease. Therefore, we tested the hypothesis that malnutrition prolongs the duration of *Escherichia coli* heat-stable enterotoxin-induced rat jejunal secretion. At weaning, rats were separated into two groups: malnourished rats were fed 50% of the previous day's intake of the fully fed control group. After approximately 2 wk of pair feeding, when malnourished rats weighed $\leq 60\%$ of the full fed control group, we measured the secretory response to heat-stable enterotoxin in ligated jejunal loops. Toxin-induced secretion was equal in both groups until 30 min incubation time, after which net secretion continued to increase in the malnourished group but decreased in the fully fed group. Jejunal brush border membranes prepared from malnourished and fully fed rats demonstrated similar heat-stable enterotoxin receptor density, avidity of binding and guanyl cyclase activation. In both groups, radiolabeled toxin injected into *in situ* jejunal loops was converted into an altered radioligand unable to bind to brush border membranes. However, in malnourished rats, there was both increased appearance of two additional radioligands that still retained their ability to bind to brush border membranes and persistence of biologically active unlabeled toxin as measured in the suckling mouse bioassay. Our studies demonstrate that reduced or delayed inactivation of heat-stable enterotoxin, with continued presence of active toxin species, may contribute to prolonged secretion in the jejunum of malnourished rats. (*Pediatr Res* 31: 228–233, 1992)

Abbreviations

ETEC, enterotoxigenic *Escherichia coli*
ST_a, *Escherichia coli* heat-stable enterotoxin
BBM, brush border membrane
K_a, apparent affinity constant of binding
FF, fully fed control group
MAL, malnourished group
cGMP, cyclic GMP

infant mortality and an estimated five million deaths per year in children under 5 y of age (1–5). Malnutrition is a major coexisting health problem in children in these developing areas, with an estimated prevalence of 30–90% (6–8). These two problems appear to be mutually aggravating; recurrent episodes of diarrhea adversely affect the nutritional status of children, and diarrheal disease may be more frequent and more prolonged in malnourished children (6–11).

Infection with ETEC is among the most common causes of endemic childhood diarrhea (2, 12–14). These ETEC elaborate several enterotoxins including ST_a, which is a small polypeptide that is able to activate guanylate cyclase, induce intestinal secretion, and cause diarrheal disease (15–17).

The aim of our study was to determine the effect of malnutrition on ST_a-mediated intestinal secretion. To test our hypothesis that the malnourished rat was more responsive to ST_a, we compared the *in situ* secretory response to ST_a in the jejunum of malnourished and fully fed rats. We also examined potential mechanisms that could account for increased responsiveness to ST_a in the malnourished rat. To do this, we compared 1) ST_a-binding, 2) ST_a-induced guanylate cyclase activation, and 3) inactivation of ST_a in the jejunum of the malnourished and fully fed rat.

MATERIALS AND METHODS

ST_a and 4-Tyr-¹²⁵I-ST_a. ST_a from *E. coli* strain 18D was purified as described by Staples *et al.* (18). Na¹²⁵I (>350 mCi/mL) was obtained from Amersham (Arlington Heights, IL) and was used to radioiodinate pure ST_a by a lactoperoxidase method (19). 4-Tyr-¹²⁵I-ST_a was purified by HPLC (19).

Animals. Twenty- to 21-d-old Sprague-Dawley rats (10–11 pups/litter), with mothers, were obtained from Zivic Miller Laboratories (Zelienople Park, PA). Animals were housed at the University of Cincinnati, and the experimental protocol was approved by the Institutional Animal Care and Use Committee. This facility is accredited by the American Association for Accreditation of Laboratory Animal Care. Rats were housed in wire-mesh cages to prevent coprophagia and were maintained in 22°C temperature-controlled rooms with 12-h alternating light-dark cycles. They were allowed water *ad libitum* throughout the study. All rats were fed standard laboratory food from hanging metal food baskets.

Animals were rendered malnourished using a modification of the pair-feeding technique described by Sherman *et al.* (20). Rat pups were fed *ad libitum* for the first day after weaning and were then randomly assigned to either the FF or the MAL. The group of animals assigned to MAL were each day allotted 50% of the average intake for the previous day for the group of animals in FF. Our studies were performed when the average body weight of MAL was approximately 60% of that of FF.

***In situ* intestinal secretion.** After intraperitoneal sodium pen-

Diarrheal disease is one of the most important health problems in developing areas of the world, accounting for up to 50% of

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tobarbital anesthesia, one or two sections of intestine beyond the ligament of Treitz of approximately 10 cm (proximal jejunum) were isolated with surgical ligatures as previously described (21). Pure ST_a (10 µg/mL) in 1 mL of saline or an equal volume of ST_a-free saline was injected into each loop with a 30-gauge needle. After *in situ* incubation for 15–240 min, the abdomen was reopened and the ligated loop was removed and weighed intact. The loop was opened, milked dry of accumulated fluid, and weighed empty. Net fluid accumulation was calculated as the difference between the weight intact and the weight empty. Secretion is expressed as fluid accumulation in an ST_a-containing loop minus fluid accumulation in a control (ST_a-free) loop. The data were expressed in µL/cm intestinal loop length. Expressing the data in mL/g intestinal loop weight gave similar results.

To evaluate the inactivation of the ST_a remaining in the secreted fluid, some animals were injected with 4-Tyr-¹²⁵I-ST_a (50–250 pM) and the secreted fluid was harvested at various times as previously described (22). Radiolabeled ST_a remaining in secreted fluid was characterized by HPLC and by the ability to rebind to rat jejunal BBM as previously described (22). In addition, biologic activity of unlabeled ST_a in secreted fluid was determined in the suckling mouse assay as previously described (22, 23).

BBM preparation. Jejunal BBM were prepared using a modification of the divalent cation precipitation technique described by Schmitz *et al.* (24) as previously validated in our laboratory (21). After preparation, BBM were promptly frozen in a –80°C freezer and stored for 1–3 wk. Sucrase enrichment was comparable for both groups (14.9 ± 0.69- and 16.8 ± 3.7-fold increase over jejunal homogenate for MAL and FF, respectively). Protein concentration was measured by the method of Lowry *et al.* (25) using BSA as the standard. DNA was quantitated by the method of Brunk *et al.* (26).

Binding of ST_a to BBM. A competitive inhibition of binding assay was used to characterize the binding of ST_a to BBM as previously described (21, 22). Briefly, BBM (60–90 µg) were incubated with a constant amount of ¹²⁵I-ST_a (approximately 45 000 cpm) and varying amounts of unlabeled ST_a (0.05–1000 ng) in 0.1 M sodium acetate, pH 4.8, with 0.15% BSA. The incubation at 37°C was terminated after 60 min by rapid suction filtration. Specific binding was determined by subtracting the binding of 4-Tyr-¹²⁵I-ST_a in the presence of >1000-fold excess unlabeled ST_a. K_a and the binding capacity were calculated by Scatchard analysis, using the least-squares-fit computer program "Ligand" as described by Munson and Rodbard (27).

Guanylate cyclase activation. ST_a-induced guanylate cyclase activation was determined using the technique described by Waldman *et al.* (28). ST_a-stimulated guanylate cyclase activity was expressed in pmol cGMP generated per mg membrane protein per min and compared with basal, unstimulated activity.

Suckling mouse assay. Biologic activity of ST_a in secreted fluid was determined in the suckling mouse assay (29). Three ND4 Swiss albino mice were inoculated by percutaneous intragastric injection with 100 µL of secreted fluid. Three h after inoculation, the mice were killed and the intestine without the stomach was removed and weighed. The ratio of gut-to-carcass weight was calculated.

Northern analysis. Total RNA was isolated from rat jejunum by the guanidine isothiocyanate method (30). An aliquot was used in a reverse transcription reaction containing an antisense primer based on the rat guanyl cyclase C cDNA sequence (31). The resulting cDNA was then used as a template for the polymerase chain reaction (Perkin Elmer Cetus, Norwalk, CT). The product of this reaction, a fragment encompassing nucleotides 54–602 of the rat guanyl cyclase C cDNA (31), was cloned into Bluescript (Stratagene, La Jolla, CA) by blunt ligation. The identity of the recombinant clone (p5'GC-C) was confirmed by sequence analysis (32).

Total RNA was then prepared from the jejunum of MAL and FF (30). For Northern blots, total RNA (20 µg) was denatured

with glyoxal, fractionated on a 1% agarose gel, and transferred to a nylon membrane by capillary action. Blots were cross-linked with short-wave UV, dried, and washed at 65°C in 1 × SSC (SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.6), 0.1% SDS. Prehybridization was performed as described by Church and Gilbert (33).

The p5'GC-C fragment of the rat guanyl cyclase C was radio-labeled with [³²P]deoxycytidine triphosphate by random primer extension (34) and hybridized under stringent conditions with final wash conditions of 0.1 × SSC, 0.5% SDS at 65°C. Autoradiography was performed at –80°C with XAR-5 film (Eastman Kodak Co., Rochester, NY) using Cronex Lightening Plus intensifying screens (New England Nuclear, Boston, MA). Densitometric analysis of the autoradiograms was obtained using a laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). The autoradiographic values were normalized based on the signal intensity seen upon hybridization with an oligonucleotide complementary to 18S ribosomal RNA (35).

Data presentation and analysis. For *in situ* experiments, each point represents the mean of three to 11 determinations. For ST_a-binding and ST_a-stimulated guanylate cyclase experiments, each point was determined in duplicate and experiments were repeated in triplicate. All data are presented as mean ± SEM. Paired comparisons were performed using the *t* test and, when appropriate, the Wilcoxon rank sum test was used for unpaired comparisons.

RESULTS

Pair feeding. Nutritional deprivation by pair feeding markedly reduced the growth of malnourished rats. The average daily weight gain during the pair-feeding period was 9.7 g for FF, whereas that for MAL was 5.7 g. The mean duration of pair feeding was 17.4 ± 0.5 d. The weight of the animals when they were killed was 174.5 ± 3.3 g for FF (*n* = 95) and 100.9 ± 3.1 g for MAL (*n* = 95), the ratio of MAL/FF weight being 0.58.

Effects of malnutrition on the intestine. As shown in Table 1, the MAL intestine weighed less and was significantly shorter than FF intestine. However, the weight of the intestine was approximately 4.5% of the total body weight in each group. Sucrase activity was significantly increased in MAL jejunum. Jejunal protein content and jejunal DNA content were reduced in MAL but these differences were not statistically significant.

In situ intestinal secretion. The time course of the net secretory response to a 10-µg/mL dose of intraluminal ST_a is shown in Figure 1. At all times, the response in the saline control loops for FF and MAL was similar (data not shown). The net secretory response to ST_a was similar in FF and MAL until 30 min of incubation, after which time net secretion continued to increase in MAL but decreased in FF. By 240 min, the last time point tested, net secretion in MAL was nearly 10-fold that of the FF (236 ± 22 versus 24 ± 15 µL/cm, respectively, *p* = 0.005). If a tissue weight basis was used to normalize the data, the difference became even more marked (5.51 ± 0.55 versus 0.25 ± 0.21 mL/g intestine, *p* < 0.001 at 240 min).

ST_a binding to BBM. To determine whether the differences in the number of ST_a receptors or avidity of ST_a binding might explain this observed difference in response to ST_a, we calculated

Table 1. Effect of malnutrition on intestinal weight, length, and sucrase activity.

	FF	MAL	<i>p</i>
Intestinal weight (g)	6.69 ± 0.30	4.01 ± 0.26	<0.001
Intestinal length (cm)	108 ± 3.9	89.4 ± 4.3	0.01
Intestinal weight/body weight	0.046 ± 0.006	0.045 ± 0.005	0.86
Sucrase activity (mU)	49.5 ± 10.2	124.5 ± 17.5	0.02
Jejunal protein (mg/cm)	4.7 ± 0.7	3.4 ± 0.3	0.10
Jejunal DNA (µg/5 cm)	626 ± 61.7	478 ± 89.6	0.17

Fig. 1. Time course of net jejunal secretion in response to a 10- μ g/mL dose of intraluminal ST_a. Net secretion in MAL (open circles) and FF (filled circles) rats was measured as described in Materials and Methods and calculated as fluid accumulation in a test loop minus fluid accumulation in a control loop. The response to a (control) injection was identical in MAL and FF rats. Data are mean $\pm$ SEM of three to 11 determinations. *, $p < 0.01$ at 120 min; **, $p < 0.001$ at 180 min; ***, $p = 0.005$ at 240 min.

the K_a of ST_a binding and number of ST_a receptors per mg protein in BBM preparations from FF and MAL. As shown in Figure 2, increasing doses of unlabeled ST_a progressively and identically inhibited the binding of ¹²⁵I-ST_a to jejunal BBM of FF and MAL. The data from these assays were used to calculate the K_a of ¹²⁵I-ST_a binding which was similar for both MAL and FF ($2.48 \pm 0.56 \times 10^9$ versus $1.81 \pm 0.50 \times 10^9$ L/mol, respectively). The number of ST_a receptors per mg BBM protein was also identical for MAL and FF ($3.34 \pm 0.99 \times 10^{12}$ versus $3.12 \pm 0.87 \times 10^{12}$, respectively). When normalized by DNA content, ST_a binding was similar in mucosal homogenates from MAL and FF (2273 ± 670 versus 2346 ± 657 cpm per μ g DNA, respectively).

Guanylate cyclase activation. To determine if increased receptor-effector coupling played a role in the increased responsiveness

of the malnourished rat jejunum to ST_a, we measured jejunal guanylate cyclase activity at basal levels and in response to increasing doses of ST_a in FF and MAL. As seen in the dose-response curves shown in Figure 3, increasing quantities of ST_a progressively and identically activated guanylate cyclase, resulting in a similar ED₅₀ (1–4 ng) and maximal stimulation (4.5-fold) in MAL and FF. Basal guanylate cyclase levels were similar in both MAL and FF (10.3 ± 2.0 versus 15.0 ± 5.5 pmol cGMP/mg/min, respectively).

ST_a receptor mRNA expression. Northern analysis was used to examine the expression of the ST_a receptor in MAL and FF. As shown in Figure 4, upon hybridization with a radiolabeled fragment of the ST_a receptor cDNA, a 3.8-kb message is seen in both MAL and FF. When quantitated by laser densitometry and normalized by 18S RNA signal, the ST_a receptor mRNA expression was equal in both groups.

Fig. 2. Effect of increasing doses of unlabeled ST_a on binding of ¹²⁵I-ST_a to BBM from MAL (open circles) and FF (filled circles) rats. Binding of ¹²⁵I-ST_a was similar in both groups. Data are mean $\pm$ SEM of three separate experiments. B/B₀, fraction of ¹²⁵I-ST_a bound in the absence of added, unlabeled toxin.

Fig. 3. Effect of increasing doses of ST_a on guanylate cyclase activation in BBM from MAL (open circles) and FF (filled circles) rats. Maximal guanylate cyclase activation and ED₅₀ were identical in MAL and FF rats. Data are mean $\pm$ SEM of three separate determinations. Data are expressed as the percentage of guanylate cyclase activation in the absence of added ST_a.

Fig. 4. Northern blot analysis of ST_a receptor expression in the jejunum of FF and MAL rats. Migration of 28S and 18S ribosomal RNA is shown on the right. For normalization of the signal intensity, hybridization with an oligonucleotide complementary to 18S ribosomal RNA was performed and is shown below.

HPLC profile and BBM binding characteristics of recovered radioactivity. A comparison of the time course of disappearance of radioactivity from MAL and FF jejunum is shown in Figure 5. In both groups, there was continued disappearance of radioactivity over time. At all time points, the percentage of remaining radioactivity was greater in MAL, but at no time point was this difference statistically significant.

To determine whether alteration of the injected radiolabeled ST_a had occurred, we evaluated the HPLC profiles of the fluid recovered from the jejunal loops of MAL and FF. Figure 6 shows a representative HPLC profile of ¹²⁵I-ST_a after a 30-min *in situ* incubation. Arrows point to those peaks that comigrate with the injected authentic 4-Tyr-¹²⁵I-ST_a. Shown above the chromatograms are the BBM binding activities of the corresponding HPLC peaks. In both FF and MAL, there was a major new peak with a shortened retention time (5 min), representing 98.4 $\pm$ 1.4% of the remaining radiotracer in FF and 61.1 $\pm$ 10.3% in MAL ($p < 0.001$). This modified radiotracer had lost the ability to bind to BBM. However, in MAL jejunum there was an increased appearance of two other peaks with retention times closer to that of authentic 4-Tyr-¹²⁵I-ST_a. These peaks, with HPLC retention times of 24 and 27 min, represented 27.7 $\pm$ 10.5 and 11.1 $\pm$ 2.4%, respectively, of the remaining radiotracer in MAL jejunum and 1.6 $\pm$ 1.4 ($p = 0.001$) and 0 $\pm$ 0 ($p = 0.02$) of the remaining radiotracer in FF jejunum. Both of these peaks (24 and 27 min) retained their ability to bind to BBM, unlike the major peak identified at 5 min. By 60 min incubation, virtually all of the remaining radiotracer in both groups had been converted to a radiolabel with a retention time of 5–10 min.

Suckling mouse assay. We evaluated the biologic activity of ST_a and its metabolites remaining in secreted fluid by determining the ability of MAL and FF jejunal fluid to stimulate secretion in the suckling mouse assay. A gut-to-carcass ratio of ≥ 0.083 was considered positive. Jejunal fluid from MAL was positive from the 30-, 60-, 120-, and 180-min time points, but negative at the 240-min incubation time point. In contrast, jejunal fluid from FF was positive at 30 min, but negative at 60 min and all subsequent time points.

DISCUSSION

A number of studies have demonstrated that there is a direct relationship between malnutrition and the severity and duration

Fig. 5. Time course of disappearance of radioactivity from the jejunum of MAL (open circles) and FF (filled circles) rats. At various times, the fluid remaining in ligated loops was harvested, homogenized in an equal volume of ice-cold trichloroacetic acid, and centrifuged for 30 min at 40 000 $\times$ *g*. Radioactivity remaining in the supernatant was compared with the amount of radioactivity initially injected into the loop and expressed as the percentage of injected cpm remaining. Data are mean $\pm$ SEM of three to 11 separate determinations.

Fig. 6. HPLC profile and BBM binding activity of radioactivity remaining after 30 min of *in situ* incubation in FF and MAL rat jejunum. Arrows point to the HPLC retention time of unmodified 4-Tyr-¹²⁵I-ST_a. A representative chromatogram from FF rats is shown on the left and from MAL rats on the right. Above each chromatogram is shown the BBM binding activity of the corresponding radioisotopic HPLC peak. Data are expressed as mean $\pm$ SEM of three separate determinations. Binding data are expressed in cpm specifically bound per 100 μ g BBM protein after a 1-h incubation at 37°C (see Materials and Methods). ND, no binding detected.

of diarrheal illness (9–11, 36). Our data provide evidence for an increased and prolonged secretory response to ST_a in the jejunum of the malnourished rat. Our pair-feeding model produced total calorie deficiency corresponding to severe malnutrition, defined as a body weight <60% of the expected weight for age (37). This degree of malnutrition produced diminished intestinal weight and length as well as changes in intestinal protein content and cellularity (DNA content). We have, therefore, where possible, normalized data in several different ways to minimize the effects of these variables.

To determine the mechanism for this prolonged secretory response to ST_a, we examined the possibility that there were malnutrition-associated increases in the avidity of ST_a binding, the number of toxin receptors, or the responsiveness of guanylate cyclase to ST_a. Our findings of identical binding of ST_a and identical dose response curves for ST_a-mediated guanylate cyclase activation in MAL and FF do not support the hypothesis that these factors are involved in the increased response of the malnourished jejunum to ST_a. We observed an increase in jejunal differentiation or maturation in MAL as measured by an increase in sucrase activity similar to that which had been reported by others (20, 38). This increase in sucrase activity also serves as a control for the binding ability of ST_a to its receptor, which is also localized to the BBM. In this model, the ability of the jejunum to absorb a saline control injection was also similar in MAL and FF. Thus, the increase in net secretion was not due to decreased basal absorption. This finding is also consistent with the presence of equally or more mature enterocytes despite the atrophic nature of the malnourished intestine (39).

The recent demonstration that guanylate cyclase is a receptor for ST_a fits with our observations of parallel ST_a binding and ST_a-induced guanylate cyclase activation in both groups (31). The possibility remains that increased intracellular accumulation of cGMP could provide an explanation for the enhanced secretory response in MAL. We did not measure the accumulation of cGMP, which would be affected by phosphodiesterase activity (degradation) as well as guanylate cyclase activity activation (production).

We also investigated the possibility that different patterns of inactivation of ST_a might explain the increased secretory response in the malnourished jejunum. In both MAL and FF, there was modification of the injected radiotracer. However, the degree of modification and inactivation of the injected radiotracer (4-Tyr-¹²⁵I-ST_a) was greater in FF. A modified, inactive radiotracer accounted for >98% of the remaining radioactivity in FF at 30 min of incubation. Two different ligands, both able to bind to BBM, together accounted for 38% of the remaining radioactivity in MAL. Although we did not determine the nature of the modification of ST_a that resulted in the generation of the distinct HPLC profiles, each of the HPLC peaks must have retained the 4-Tyr-¹²⁵I site to have been detected. We also demonstrated a correlation between the persistence of biologic activity in the suckling mouse assay and the time course of jejunal secretion in MAL and FF. We have previously demonstrated a correlation between persistent secretion, the continued presence of unmodified ¹²⁵I-ST_a, and biologic activity of fluid from the adult rat ileum in the suckling mouse assay (22). On the basis of these data, we suggest that the continued presence of active ST_a in MAL jejunum at ≥ 30 min may contribute to the increased and prolonged secretory response to ST_a.

Recently, Young and Levin (38) demonstrated that acute starvation (≤ 72 h) made the rat jejunum hypersensitive to cholinergic and other stimuli. Thus, the possibility exists that the malnourished jejunum is hypersensitive as a result of a final common effect on chloride channel secretion, regardless of the stimulus. However, our observation of a prolonged secretory response to ST_a differs somewhat from the observations of Young and Levin, who described a greater secretory response in the starved jejunum with a similar duration of secretion.

If reduced or delayed inactivation of ST_a occurs in the intestine of the malnourished human infant, this might result in more prolonged or severe diarrheal illness. Other mechanisms may also be involved in the increased secretory response to bacterial enteropathogens observed in childhood malnutrition. These mechanisms include diminished immune competence (40), vitamin A deficiency (41), mucin depletion (20), decreased gastric

acid secretion (42), and delayed intestinal repair (43). These mechanisms would further augment the increased secretory response to ETEC resulting from impaired host defense against ST_a, i.e. toxin inactivation.

In summary, antecedent malnutrition predisposes to an increased duration of diarrheal disease in children. We have studied the response of the malnourished rat jejunum to one of the most common causes of endemic diarrhea in children. We found an increased responsiveness of the malnourished rat jejunum to ST_a, and we examined several possible mechanisms to explain this increased responsiveness. Our data best support the possibility that impairment of intestinal inactivation of ST_a contributes to an increase in ST_a-mediated secretion in the malnourished rat jejunum.

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