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Mechanisms of Increased Susceptibility of Immature and Weaned Pigs to *Escherichia coli* Heat-Stable Enterotoxin

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ABSTRACT. **Pigs demonstrate an increased sensitivity and susceptibility to *Escherichia coli* heat-stable enterotoxin (ST_a) in the 1st wk of life and immediately after weaning. To determine the possible mechanisms for this increased susceptibility, we compared ST_a binding, guanylate cyclase activation, and photoaffinity cross-linking to porcine jejunal brush border membranes prepared from immature (≤1 wk of age) *versus* adult pigs as well as 3-wk-old weaned *versus* unweaned pigs. The ST_a binding capacity of immature pigs was nearly twice that of adult pigs (11.73 ± 1.52 *versus* $6.00 \pm 0.96 \times 10^{-11}$ mol/L, $p < 0.001$), and the ST_a binding capacity of weaned pigs was nearly three times greater than that of unweaned pigs (17.48 ± 2.10 *versus* $4.86 \pm 1.02 \times 10^{-11}$ mol/L, $p < 0.001$). Scatchard analysis suggested a single class of ST_a receptor, with an association of binding constant of $\sim 10^9$ L/mol at all ages. Maximum guanylate cyclase response (expressed as pmol cyclic GMP generated/mg brush border membrane protein/min) was greater in immature *versus* adult pigs (1312 ± 831 *versus* 320 ± 92, $p < 0.02$). Weaned pigs had a greater maximum guanylate cyclase activation than unweaned pigs (1126 ± 692 *versus* 624 ± 298); however, this difference was not statistically significant. Autoradiograms demonstrated specific cross-linking of ^{125}I-ST_a to a number of distinct radiolabeled bands (62, 66, 84, 92, 160, and 165 kD). There was a difference in the size and trypsin sensitivity of these radiolabeled bands as a function of age and weaning. Treatment with trypsin decreased the intensity of the 160 to 165-kD bands while increasing the intensity of the 62- to 66- and 84- to 92-kD bands. These differences in ST_a binding, guanylate cyclase activation, and ST_a receptor size may increase the susceptibility of pigs during the 1st wk of life and at weaning to ST_a-mediated diarrheal disease. (*Pediatr Res* 29: 424–428, 1991)**

Abbreviations

ST_a, *Escherichia coli* heat-stable enterotoxin
BBM, brush border membrane
K_a, affinity constant of binding

ST_a are a family of related polypeptides that bind to an intestinal receptor (1), stimulate membrane-bound guanylate cyclase (2, 3), and thereby induce intestinal secretion (4). This process has been studied in human and rat intestine, and in both there is an increased density of BBM receptors for ST_a in the immature intestine (5, 6). This coincides with the period of increased susceptibility to ST_a-induced diarrheal disease that occurs in early life in humans (7, 8). *Escherichia coli* that produce ST_a are also natural pathogens for the pig, causing clinically significant diarrheal disease (9, 10). Stevens *et al.* (11) have described two periods of increased porcine responsiveness to ST_a: *1*) during the 1st wk of life (immature animals) and *2*) immediately after weaning. These observations provide a unique window of opportunity to investigate the possible mechanisms for increased susceptibility and host responsiveness to enterotoxigenic *E. coli* in the pig.

The aim of our studies was to determine if increased ST_a binding and guanylate cyclase activation correlates with the increased susceptibility to ST_a-mediated diarrheal disease found in immature and weaned pigs. Our hypothesis was that the immature pig has an increased binding capacity for ST_a and increased ST_a-induced guanylate cyclase activation compared with the adult. Similarly, we hypothesized that the weaned pig has an increased binding capacity for ST_a and an increased guanylate cyclase activation compared with its unweaned counterpart. As a related aim, we wished to determine if there were differences in the cross-linking pattern of ^{125}I-ST_a to BBM prepared from pigs of different ages.

MATERIALS AND METHODS

Porcine intestine. Animals were housed at the Midwest Area National Animal Research Center, Ames, Iowa, 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 Lab Animal Care. Suckling pigs were raised and cared for in the same manner as the control pigs previously reported (12). They were housed with their mothers before weaning, and at weaning were separated from their mothers into different rooms, where they received solid food and water (12). Pigs were considered immature if they were ≤1 wk of age, and adult if they were ≥6 mo of age. Several groups of pigs were studied: ages ≤1 wk ($n = 8$), weaned (3 wk old, weaned 3 d previously; $n = 5$), unweaned (3 wk old, unweaned; $n = 5$), and adult ($n = 3$). For some studies, additional pigs age 14 d ($n = 2$) and 6 wk ($n = 2$) were used. Their intestines were removed, rinsed with normal saline, and immediately frozen at −70°C. All experiments were carried out on intestinal segments taken from the proximal third of pig small bowel.

ST_a. ST_a was purified from *E. coli* strain 18D as previously described (13). Pure ST_a was radioiodinated using a lactoperoxidase method, and 4-tyrosine-^{125}I-ST_a was purified by an HPLC technique (14).

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Chemicals. ^{125}I-sodium (>350 mCi/mL) was obtained from Amersham (Arlington Heights, IL). Enzymatic reagents were obtained from Sigma Chemicals Co. (St. Louis, MO), and all other chemicals were reagent grade.

Protein. All protein determinations were performed using the method of Lowry *et al.* (15), using a BSA standard.

Jejunal BBM. BBM were prepared using a modification of the divalent cation precipitation technique described by Schmitz *et al.* (16). Intestinal mucosa was scraped from 5 to 10-cm thawed segments of proximal jejunum. The mucosa obtained was homogenized in a 250 mM sucrose/1 mM EDTA/1 mM DTT/50 mM Tris/HCl buffer solution (pH 7.3) for 2 min in a Sorvall Omni Mixer (Dupont Instruments, Wilmington, DE). Calcium chloride, to a final concentration of 10 mM, was added to the homogenate, and this mixture was stirred for 20 min at 4°C. This suspension was centrifuged at 2000 × *g* for 10 min, and the pellet containing nuclear fragments and mitochondria was discarded. The supernatant was then centrifuged at 17 500 × *g* (to precipitate BBM), and the resultant pellet was washed in 5 mM Tris buffer, pH 7.6, centrifuged at 17 500 × *g*, and then resuspended in 5 mM Tris buffer, pH 7.6.

Disaccharidase determination. To compare BBM preparations from pigs of different ages and feedings, we first validated the similarity of BBM preparations by determining the enrichment of disaccharidase activity from homogenate to the BBM preparation. All specimens tested had a 15.0 ± 4.4-fold increase in disaccharidase activity. All intestinal specimens obtained from animals greater than 5 d old were assayed for sucrase activity by the method of Dahlqvist (17). Specimens obtained from animals 5 d of age or less were assayed for lactase activity using the method of Dahlqvist as modified by Koldovsky *et al.* (18).

ST_a binding. For determination of the K_a and receptor number, a competitive inhibition of binding format was used as previously described (1, 6). Briefly, BBM were incubated with ^{125}I-ST_a in 12 × 75 mm glass tubes. Sodium acetate buffer (100 mM, pH 4.8) with 0.15% BSA was added to each tube to bring the total volume to 1 mL. Tubes were incubated at 37°C for 1 h, and the reaction was terminated by rapid suction filtration using a Millipore multichamber sampling manifold (Millipore Corp., Boston, MA) and Whatman GF/B glass fiber filters (Whatman Ltd., Maidstone, England) soaked in 0.3% poly(ethylene)imine (Eastman Kodak, Rochester, NY). Filters were rinsed twice with 3 mL of ice cold 50-mM sodium acetate, pH 4.8, and counted in a gamma-scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). These counts represented total counts of ^{125}I-ST_a bound to its BBM receptor. Aliquots of BBM (25 μg) were incubated with 100 000 cpm of ^{125}I-ST_a (45 pM) in the presence of increasing concentrations of native ST_a (0.025–500 nM). Specific binding was determined by subtracting total counts bound in the presence of excess (500 nM) native ST_a (nonspecific binding). The apparent K_a and receptor binding capacity were determined using the computer program Ligand as described by Munson and Rodbard (19).

Guanylate cyclase activation. ST_a-induced guanylate cyclase activation was determined using the technique described by Waldman *et al.* (20). BBM were incubated at 32°C with guanosine triphosphate and guanosine triphosphate-regenerating mixture, in the presence and absence of ST_a. Cyclic GMP generated was assayed by an RIA technique previously validated (4). ST_a-stimulated guanylate cyclase activation was expressed in pmol cyclic GMP generated/mg BBM protein/min.

Photoaffinity cross-linking of ST_a to BBM. Porcine BBM (100 μg) were incubated for 60 min at 37°C in 100 mM sodium acetate buffer (pH 4.8) with 800 000 cpm (360 pM) of radiolabeled ST_a, in the presence and absence of 2 μg (1 μM) of native ST_a in a total reaction mixture of 1 mL. This reaction mixture was microfuged for 10 min at 4°C, and the pellet resuspended in 10 mM sodium phosphate buffer solution (pH 7.2). Cross-linking of radiolabeled ST_a, bound to its BBM receptor, was then accomplished using the heterobifunctional photoaffinity cross-linking agent N-hydroxysuccinimidyl-4-azidobenzoate. The mixture was left in the dark on ice for 20 min, then pulsed with an 8-min flash of short-wave UV light. The reaction was then terminated by the addition of 50 mM Tris buffer (pH 7.4). These cross-linked porcine BBM were boiled in 2% SDS and 5% 2-mercaptoethanol before analysis by SDS-PAGE using a 5% stacking gel and 10% resolving gel (21). Gels were stained with Coomassie blue, dried, and exposed to Kodak XAR-2 film for 72 h at −70°C.

DATA presentation and statistical analysis. Individual points were determined in duplicate in the ST_a binding experiments and in triplicate in the guanylate cyclase activation experiments. Binding capacity at different ages was compared using the *t* test. Maximum guanylate cyclase activity was compared at different ages using the nonparametric Wilcoxon rank sum test, due to the significant variability of measurements. Statistical significance was assumed at $p < 0.05$. Data were expressed as mean ± SEM.

RESULTS

ST_a binding. Specific binding of 100 000 cpm of ^{125}I-ST_a increased in a linear manner when BBM protein concentrations ranging from 5 to 80 μg were added to 1 mL of 100 mM sodium acetate buffer (pH 4.8). Specific binding increased linearly with incubation time from 15 min to 45–60 min, at which time a plateau was reached. Therefore, an incubation time of 60 min and a protein concentration of 25 μg were used in all experiments. Binding of ^{125}I-ST_a to its intestinal BBM receptor was progressively inhibited, in all specimens tested, by increasing doses of native ST_a (Fig. 1). A Scatchard plot (19) of these competitive inhibition of binding curves revealed a linear plot at all ages, indicating a single species of ST_a receptor (data not shown). The K_a was the same in all animals tested (approximately 10^9 L/mol); however, as shown in Figure 2, the ST_a binding capacity of porcine BBM was highest during the 1st wk of life and just after weaning. The ST_a binding capacity of immature pigs was twice that of adult pigs (11.73 ± 1.52 *versus* 6.00 ± 0.96 × 10^{-11} mol/L, $p < 0.001$). The ST_a binding capacity of 3-wk-old weaned pigs was more than three times the ST_a binding capacity of 3-wk-old unweaned pigs (17.48 ± 2.10 *versus* 4.86 ± 1.02 × 10^{-11} mol/L, $p < 0.001$). After weaning, the ST_a binding capacity decreased by 6 wk to levels similar to that seen in the adult animals (8.60 ± 0.60 versus 6.00 ± 0.96 × 10^{-11} mol/L, p = NS). Changes in ST_a binding activity did not correlate with changes in sucrase activity. As also shown in Figure 2, sucrase activity increased from the 1st wk of life until the time of weaning (3 wk unweaned). Sucrase activity then decreased to adult levels

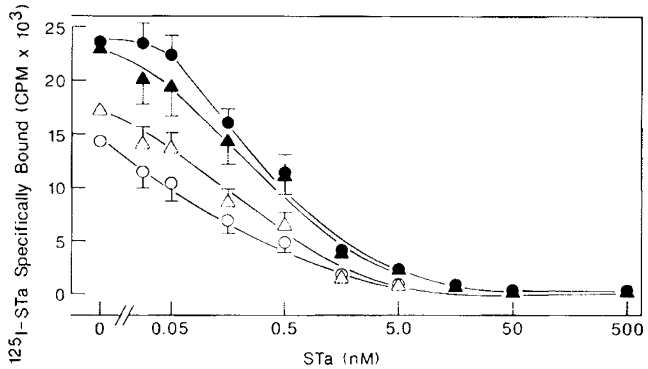

Fig. 1. Competitive inhibition of ^{125}I-ST_a binding to jejunal BBM by increasing doses of native ST_a. Jejunal BBM from immature (≤1-wk-old) (●), adult (○), 3-wk-old weaned 3 d before sacrifice (▲), and 3-wk-old unweaned (△) pigs were incubated for 60 min in 1 mL of 100 mM sodium acetate buffer (pH 4.8) at 37°C with 100 000 cpm of ^{125}I-ST_a. Binding of ^{125}I-ST_a was determined as described in Materials and Methods. Results are mean ± SEM of three to eight separate determinations.

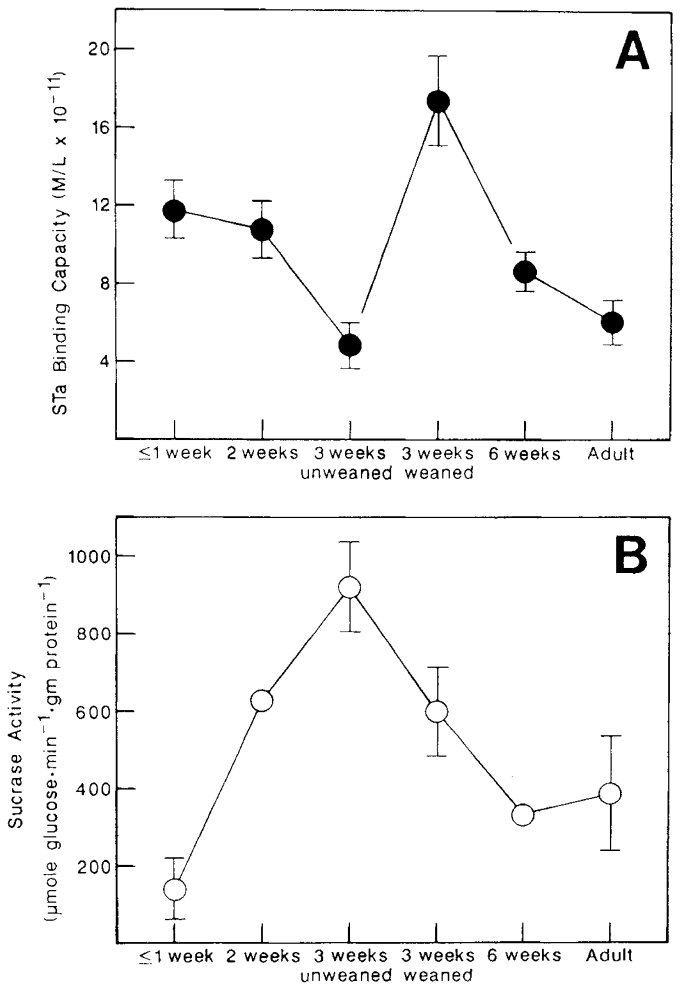

Fig. 2. ST_a binding capacity (*A*) and sucrase activity (*B*) in BBM prepared from pigs of various ages. ST_a binding activity was calculated by Scatchard analysis of competitive inhibition of binding experiments (see Fig. 1) using the computer program Ligand (19). Sucrase activity was measured as described in Materials and Methods. Results are mean ± SEM of three to eight separate determinations, except for sucrase activity at 14 d and 6 wk, where results are the mean of two determinations.

at the time of weaning (3 wk weaned), a time when ST_a binding activity was highest.

Guanylate cyclase activation. ST_a stimulated BBM guanylate cyclase activation in a dose-dependent manner in all specimens tested. Guanylate cyclase stimulation increased linearly with increasing concentrations of BBM protein added, between 2.5 and 20 μg. Increasing guanylate cyclase activation was also directly related to increasing incubation time between 1 and 10 min, at which point a plateau was reached. Therefore, all experiments were performed with an incubation time of 3 min, at 32°C, with 10 μg of BBM protein added. The dose-response curves generated by these experiments are shown in Figure 3. Sensitivity to ST_a stimulation was similar at all ages tested, with a concentration of ST_a of approximately 36 nM required to generate a half maximal response. Maximum guanylate cyclase stimulation varied greatly from animal to animal within each age group; however, the average maximal response in immature pigs was 4-fold greater than the average maximal response in adult animals (1312 ± 831 *versus* 320 ± 92 pmol/mg BBM protein/min, $p < 0.02$). The average maximal stimulation of guanylate cyclase was greater in 3-wk-old weaned pigs than in 3-wk-old suckling pigs (1126 ± 692 *versus* 624 ± 298 pmol/mg BBM protein/min); however, this difference did not achieve statistical significance because of interanimal variability.

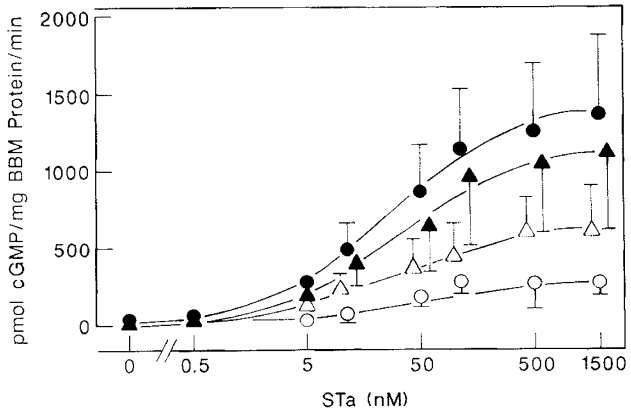

Fig. 3. Effect of increasing concentrations of ST_a on guanylate cyclase activation in membranes prepared from immature (≤1-wk-old) (●), adult (○), 3-wk-old weaned 3 d before sacrifice (▲), and 3-wk-old unweaned (△) pigs as described in Materials and Methods. Sensitivity to ST_a stimulation was similar at all ages tested, with ED_{50} at approximately 36 nM of ST_a added, for all pigs.

Photoaffinity cross-linking. Two separate BBM preparations from pigs at ages 1 d, 5 d, 7 d, 14 d, 6 wk, and adult, as well as three separate BBM preparations from 3-wk-old weaned and 3-wk-old unweaned pigs, were subjected to photoaffinity cross-linking, SDS-PAGE, and autoradiography. As shown in Figure 4, a number of distinct, specifically radiolabeled bands are apparent on these autoradiograms. In the adult, there is a prominent band at approximately 66 kD, whereas in the immature (5-d-old) pig there are bands of similar intensity at approximately 62, 84, and 160 kD (Fig. 4*A*). This pattern also occurred in other pigs <3 wk old (data not shown). Nonspecific cross-linking to a 43-kD band also occurred at all ages. At 3 wk of age, both the immature and adult patterns were present (Fig. 4*B*). The immature pattern was present in two out of three weaned pigs tested, and the adult pattern was present in two of the three unweaned pigs evaluated. There were also small but consistent differences in the apparent molecular weight of the proteins to which ST_a was cross-linked in different age animals (Figs. 4 and 5). As shown in Figure 4*B*, radiolabeled bands are seen at approximately 62 kD in the unweaned pig and at approximately 66, 92, and 165 kD in the weaned pig. These differences were confirmed in separate experiments in which the 62- to 66-kD bands from adult and 1-d-old animals did not comigrate in a single lane of a SDS-PAGE (data not shown).

To further evaluate whether these autoradiographic bands represented larger protein complexes, we subjected the BBM to 0.01% trypsin for 10 min at room temperature, followed by photoaffinity cross-linking with ^{125}I-ST_a. As shown in Figure 5, trypsin treatment had virtually no effect on adult BBM. A prominent 66-kD band is apparent on both trypsin-treated and untreated lanes. However, at all other ages, there was a trypsin effect. In the 1-d-old pig, the untreated lane has specifically radiolabeled bands at approximately 160 and 84 kD. After treatment with trypsin, there is a decreased intensity of the 84-kD band and appearance of a prominent 62-kD band. In the 3-wk-old weaned pig, the untreated lane has specifically radiolabeled bands of similar intensity at approximately 165, 92, and 66 kD. After treatment with trypsin, there is decreased intensity of the 165-kD band and more prominent labeling of the 92- and 66-kD bands. A similar pattern with slightly different molecular weight bands is seen after trypsin treatment of the 3-wk-old unweaned pig; in this lane, there is more prominent radiolabeling of bands at 84 and 62 kD.

DISCUSSION

We have shown that ST_a binds to porcine jejunal BBM and activates guanylate cyclase in both immature and adult pigs. Our

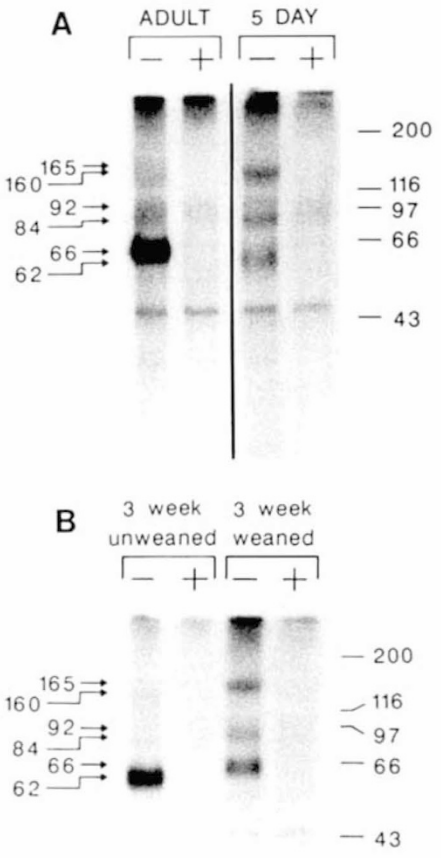

Fig. 4. Representative autoradiograms of SDS-PAGE with ^{125}I-ST_a cross-linked to porcine jejunal BBM of various age pigs. Cross-linking with N-hydroxysuccinimidyl-4-azidobenzoate was performed after incubation of ^{125}I-ST_a in the presence (+) and absence (−) of excess native ST_a, as described in Materials and Methods. *A*, adult and 5-d-old pigs. *B*, weaned and unweaned pigs, age 3 wk.

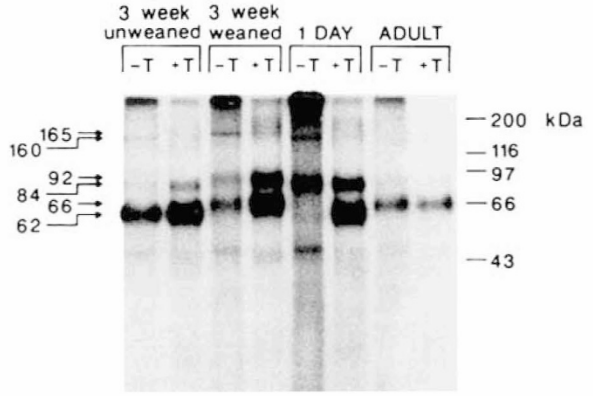

Fig. 5. Representative autoradiogram of SDS-PAGE with ^{125}I-ST_a cross-linked to porcine jejunal BBM of various age pigs after treatment with trypsin (+*T*) or trypsin-free buffer (−*T*). Cross-linking of ^{125}I-ST_a to BBM with N-hydroxysuccinimidyl-4-azidobenzoate is shown only in the absence of excess native ST_a (see Fig. 4).

data demonstrate that ST_a binding capacity is nearly 2-fold greater, and ST_a-induced guanylate cyclase activation is nearly 4-fold greater in the jejunal BBM specimens obtained from immature pigs. These differences parallel changes that have been observed in rats and humans (5, 6, 22), and may increase the susceptibility of immature pigs to ST_a-mediated diarrheal disease. Weaned pigs demonstrated more than a 3-fold greater binding capacity for ST_a than their unweaned 3-wk-old counterparts. Maximum guanylate cyclase activation in weaned pigs was nearly 2-fold greater than the average maximal stimulation seen in suckling pigs, although this difference did not achieve statistical significance due to significant interanimal variability. It is possible that confounding variables, such as stress, may alter the response to ST_a in weaned pigs. The stress associated with forced separation of immature animals from their dams at weaning may induce hormonally mediated changes in BBM (23). Stress might similarly affect the ST_a-mediated responsiveness of guanylate cyclase.

Other factors may also be involved in the increased sensitivity of weaning pigs to ST_a-mediated diarrheal disease. The data of Miller *et al.* (24) and of Hall and Byrne (25) suggest that weaning diarrhea is due to host factors that induce a cell-mediated immune response to dietary antigens and result in damage to the small intestinal mucosa at the time of introduction of a pelleted meal. Sarmiento *et al.* (26) suggest that the change in dietary antigens at weaning may not be required for the induction of diarrhea, but may modestly increase its severity. However, Hampson (27) found that the changes occurring in the intestinal mucosa at weaning may be present in the absence of diarrhea, and therefore postulates that dietary antigens play little role in weaning diarrhea. Lönnroth *et al.* (28) have identified an antisecretory factor in sow's milk that is protective against infection with ST_a-producing *E. coli*, as long as the animal is still suckling. Weaning deprives animals of this factor, and they therefore have an increased incidence of ST_a-mediated diarrhea.

Although it is likely that there are multiple factors contributing to the increased susceptibility of pigs to enterotoxigenic *E. coli* at weaning, and no one factor will explain this predilection, our data suggest a unifying concept. The variable guanylate cyclase response to ST_a that we observed may signify a predisposition to increased secretion in a selected population of pigs (hypersecretors). This predisposition might be variably expressed on the basis of permissive host factors, including BBM changes induced by dietary antigens (24, 25), or by environmental factors, including decreased maternal antisecretory factor (28) or stress. The hypersecretors, when exposed to ST_a, have increased guanylate cyclase activation and intestinal fluid secretion, and therefore an increase in sensitivity to ST_a-mediated diarrheal disease. This increased susceptibility might be augmented or more fully expressed by the action of any of these host or environmental factors.

Autoradiograms of BBM that were photoaffinity-labeled with ^{125}I-ST_a and separated by SDS-PAGE show different patterns with age. In the adult, there is a prominent band at approximately 66 kD. In contrast, in the immature pig there are several radiolabeled bands. In the 1-d-old pig there is no band corresponding to the 66-kD band seen in the adult. However, after treatment with trypsin, there is appearance of a new 62-kD, but not a 66-kD, radiolabeled band. A 160-kD band is seen in the 1-d-old and 3-wk-old unweaned pigs, whereas a slightly larger, 165-kD band is seen in the weaned pigs. However, after treatment with trypsin, the intensity of all of these bands diminishes and there is increased or new radiolabeling of smaller bands (62 kD in the 1-d-old, 62 and 84 kD in the unweaned, and 66 and 92 kD in the weaned). The higher (84 to 92 and 160 to 165-kD) bands were most prominent in immature pigs and 62- to 66-kD bands were most prominent in older pigs. Thus, there appears to be a difference in the size of the radiolabeled bands, the relative proportion of certain size classes of radiolabeled bands, and the degree of trypsin sensitivity of these bands in various age pigs. On the basis of these data, we suggest that there are structural changes with age and weaning at the ST_a binding site. It is possible that the two smaller molecular weight species are subunits of the larger 160- to 165-kD complex. Within a given size

class of proteins to which ST_a was specifically cross-linked, *e.g.* 62–66 kD, there were small but reproducible differences in apparent molecular weight as a function of age and weaning. These differences in apparent molecular weight may represent changes in glycosylation of these proteins.

In summary, we have demonstrated an increased binding capacity and guanylate cyclase activation of proximal jejunal segments of immature *versus* mature pigs, and an increased binding capacity of weaned *versus* suckling pigs. We have also shown a difference in the autoradiograms obtained from ^{125}I-ST_a photoaffinity-labeled BBM from these animals, implying structural changes in the BBM receptor with age. These results may help explain the increased susceptibility of immature and weaned animals to ST_a-mediated diarrheal disease. This model can be used for future studies addressing the regulation of factors that cause increased host responsiveness or increased susceptibility to diarrhea caused by enterotoxigenic *E. coli.*

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