Adequate oral threonine is critical for mucin production and gut function in neonatal piglets

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1Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta; 2Department of Biochemistry, Memorial University of Newfoundland, St. John’s, Newfoundland; and 3The Research Institute, The Hospital for Sick Children, and Departments of Paediatrics and Nutritional Sciences, University of Toronto, Toronto, Ontario, Canada

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Law GK, Bertolo RF, Adjiri-Awure A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production and gut function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol 292: G1293–G1301, 2007. First published January 18, 2007; doi:10.1152/ajpgi.00221.2006.—In previous experiments, we found that the threonine requirement of neonatal piglets fed parenterally was 40% of that when fed intragastrically; we hypothesized that much of the oral supply of threonine is being used for mucin production. To investigate this hypothesis, intragastrically fed 2-day-old piglets were fed one of three treatments for 8 days: 1) a threonine-adequate diet (IG-A; 0.6 g threonine · kg⁻¹ · day⁻¹ fed intragastrically); 2) a threonine-deficient diet (IG-D; 0.1 g threonine · kg⁻¹ · day⁻¹ fed intragastrically); or 3) a threonine-deficient diet with adequate threonine delivered parenterally (IV-A; 0.5 g threonine · kg⁻¹ · day⁻¹ fed parenterally plus 0.1 g threonine · kg⁻¹ · day⁻¹ fed intragastrically). IG-D piglets experienced higher nitrogen excretion, higher plasma urea, and lower plasma threonine concentrations versus both of the other groups (P < 0.05), indicating profound threonine deficiency. Mucosal mass and total crude mucin content were lower in the colons of IG-D pigs (P < 0.05). Histopathological analysis showed lower numbers of acidic mucin-producing goblet cells in the duodenum and ileum of IG-D pigs. In IG-D pigs, acidic mucin subtypes were lower in the small intestine but higher in the colon, which corresponded with persistent diarrhea. The parenteral supply of threonine was adequate to maintain most outcome parameters, although IV-A pigs did have smaller colonic goblet cells with more acidic mucins compared with IG-A pigs. Overall, our results suggest that adequate dietary threonine was critical in the production of mucus and that a parenteral threonine supply can ameliorate most of the symptoms of oral threonine deficiency.

IN A PREVIOUS STUDY, we (3) determined that the threonine requirement for piglets of ~8 days of age was 0.20 g · kg⁻¹ · day⁻¹ when fed intravenously and 0.55 g · kg⁻¹ · day⁻¹ when fed intragastrically. These results suggested that a substantial portion of the oral threonine requirement is used by the healthy gut and is not required when the gut is relatively inactive and atrophied, as during parenteral feeding (4). Recent studies (24, 27, 29, 30) have demonstrated that the portal-drained viscera, metabolically dominated by the small intestine, extracts 60–90% of dietary threonine on the first pass, whereas extraction of other essential amino acids is limited to about a third. The vast majority of this threonine is incorporated into mucosal proteins and only 2–9% is oxidized (24). The disproportionate requirement for threonine by intestinal tissues has significant nutritional implications, especially in situations of altered gut metabolism.

The difference between enteral and parenteral threonine requirements can be explained by threonine’s importance in the maintenance of the mucus lining of the gastrointestinal tract (3, 17). Threonine is an integral constituent of intestinal mucin proteins (17, 31). Mucin proteins provide the structural backbone of the mucus gels that provide lubrication and protection from pathogens (25). Without a well-formed mucus gel layer, the underlying mucosa is more susceptible to attack by bacteria such as Escherichia coli (15, 25). We therefore reasoned that mucin production would be impaired by restricting the dietary intake of threonine.

Another potential reason for the difference in threonine requirement between oral and parenteral nutrition could be due to the route of nutrient delivery. There is growing evidence that mucosal cells preferentially recruit amino acids from either the luminal or arterial supply depending on dietary and physiological conditions (23, 26, 30). It is feasible that the exclusively intravenous supply of threonine during parenteral nutrition is not available to gut mucosal cells to synthesize mucin. Therefore, this study investigated the relationship between threonine and gut mucin production to elucidate the difference in intravenous and oral threonine requirements. The objectives of the following experiment were to evaluate the effect of amount and route of dietary threonine on the quantity, location, and type of gut mucins. Specifically, the effect of an inadequate supply of threonine on gut mucins was compared with an adequate supply of threonine. Also, the difference in gut mucins was compared between threonine supplied orally versus intravenously.

MATERIALS AND METHODS

Animals and surgery. Twenty-one intact male Yorkshire piglets (2 days of age, 1.8 ± 0.3 kg), obtained from the minimal disease herd at the University of Alberta, were randomly assigned to one of three enteral dietary treatments: a threonine-adequate diet (IG-A; 0.6 g threonine · kg⁻¹ · day⁻¹ fed intragastrically); a threonine-deficient diet (IG-D; 0.1 g threonine · kg⁻¹ · day⁻¹ fed intragastrically); or a threonine-deficient diet with adequate threonine delivered parenterally (IV-A; 0.5 g · kg⁻¹ · day⁻¹ fed intravenously). All procedures used in this study were approved by the Faculty Animal Policy and Welfare Committee of the University of Alberta. Upon arrival, piglets were anesthetized, and catheters were placed in the stomach for intragastric feeding, in the left femoral vein for blood sampling, and in the left jugular vein for the intravenous infusion of saline and/or threonine.

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using previously described procedures (3); the femoral catheter was advanced to the inferior vena cava just caudal to the heart, and the jugular catheter was advanced to the superior vena cava just cranial to the heart.

Elemental and complete (except for threonine) diets (32) were fed via the gastric catheter continuously for 8 days following surgery. Vitamins (MVI Paediatric, Rhone-Poulenc Rorer, Montreal, PQ, Canada), minerals (Micro+6 concentrate, Sabex, Boucherville, PQ, Canada), and lipids (20% Intralipid, Fresenius-Kabi, Stockholm, Sweden) were added to the sterile diet solutions immediately before they were used. Following surgery, all piglets were adapted to diet infusions as previously described (3). Piglets were weighed each morning, and the infusion rates were adjusted accordingly. Diets were administered through a tether-swivel system (Alice King Chatham Medical Arts, Los Angeles, CA) using pressure-sensitive infusion pumps. The infusion regimen was designed to supply all nutrients required by piglets (32), and the targeted intakes were as follows: 15 g amino acids \( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) and 1.1 MJ metabolizable energy \( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \), with glucose and lipids each supplying 50% of nonprotein energy.

The amino acid pattern (except threonine), which was similar to that of a commercial parenteral nutrition solution that is based on human milk protein (Vaminolact, Fresenius-Kabi), consisted of the following (in mg/g total L-amino acids): 92 alanine, 61 arginine, 61 aspartic acid, 15 cysteine, 105 glutamic acid, 33 glycine, 31 histidine, 46 isoleucine, 104 leucine, 56 lysine, 19 methionine, 32 phenylalanine, 83 proline, 56 serine, 5 taurine, 21 tryptophan, 27 tyrosine, 46 valine, and 83 proline. In addition, 83 proline, 56 serine, 5 taurine, 21 tryptophan, 27 tyrosine,

Nitrogen retention. Nitrogen in diets and daily urine samples were determined by Kjeldahl analysis (6). Nitrogen retention (%) was equal to the nitrogen balance (the difference between total nitrogen intake from the diet and output from urine) divided by total nitrogen intake.

Plasma analyses. Plasma amino acid concentrations in daily blood samples were determined by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (5). Plasma urea concentrations in daily blood samples were determined using a spectrophotometric assay kit (Sigma Chemical, St. Louis, MO); conversion of absorbance to urea concentration used a standard curve of urea samples of known concentrations.

Isolation of crude mucin. Crude mucin (subdivided into native or undigested mucin and pronase-digested mucin) was isolated from mucosal scrapings according to modified procedures (17) of Allen (1) and Miller and Hoskins (20). Mucosal scrapings were lyophilized, and 0.5 g was weighed into a 50-ml polystyrene test tube; 25 ml of NaCl (0.15 mol/l with 0.02 mol/l sodium azide) were added and homogenized for 1 min at 4°C using a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada). Samples were centrifuged immediately at 4°C for 30 min at 12,000 g, and 16 ml of the aqueous supernatant were added to 24 ml of ice-cold ethanol. Samples were allowed to precipitate overnight at −20°C and then centrifuged at 4°C for 10 min at 1.400 g. The supernatant was decanted, and the pellet was resolubilized in 16 ml NaCl (0.15 mol/l), cooled in an ice bath, and then mixed with 24 ml of ice-cold ethanol. Samples were again allowed to precipitate overnight at −20°C and then centrifuged; this procedure was repeated until a clear supernatant was obtained. The final precipitate was resolubilized in 1 ml of distilled deionized water (ddH2O) and lyophilized.

Mucin quantification by carbohydrate analysis. Carbohydrate analysis was based upon the method of Lien (17) with modifications. Exactly 1.5 ml H2SO4 (12 mol/l) was added to 50 mg of isolated crude mucin and left to stand for 1 h at room temperature. The solution was diluted to 3 mol/l with 4.5 ml ddH2O and hydrolyzed for 1 h at 110°C. 200 µl internal standard was added (N-methylglucamine for amino sugars and myo-inositol for neutral sugars, 10 mg/ml), and a 1-ml aliquot of the acid hydrolysate was cooled in an ice bath and made basic with 700 µmol/l of concentrated ammonium hydroxide. Of this, 100 µl were taken, and 1 ml of sodium borohydride (30 mg/ml in anhydrous dimethylsulfoxide) was added. The Ring-opening reduction reaction was allowed to occur for 90 min at 40°C. Excess sodium borohydride was decomposed with 5 ml ddH2O and cooled to room temperature. Alditol acetates were then extracted into 4 ml dichloromethane by vigorous shaking and removal of the upper aqueous layer. Acetates were washed twice with 4 ml ddH2O and dried under nitrogen. Alditol acetates were redissolved in 1 ml dichloromethane, and 0.5 µl were injected onto the gas chromatography column. The column used was a DB-17 fused silica capillary column (0.25 mm inner diameter x 30 m), using He (1.5 ml/min) as the carrier gas. The injector temperature was set to rise from 60 to 270°C at 150°C/min and maintained for 20 min. The oven temperature was set to rise from 50 to 190°C at 30°C/min, maintained for 3 min, then set to 270°C at 5°C/min, and maintained for 5 min. The flame ionization detector temperature was set at 270°C.

Analysis of histological samples. Portions of the intestinal tract of ~2 cm in length were taken from the duodenum, midjejunum, ileum, and proximal colon. Samples were submerged in fresh chilled fixative, processed for histological analysis, and removed. The remaining jejunum was tied off into three equal lengths and removed. The colon was tied off at its midpoint, uncoiled, and separated as proximal and distal sections. Sampling of each section for histology, removal of luminal contents, scraping of mucosa, and freezing of resulting samples were all performed as described for the duodenum.
**(Bouin’s solution)** for 24 h, soak rinsed several times in absolute alcohol, and then further stored (fixed) in 10% neutral buffered formalin. After fixation, longitudinal strips of the intestine were trimmed from the antimesenteric border and routinely processed (Fisher model 266 Histomatic Tissue Processor, Fisher Scientific, Pittsburgh, PA) and embedded in paraffin (Paraplast Tissue Embedding Medium, Oxford Labware, St. Louis, MO). Serial 5-μm longitudinal sections were cut on a microtome (Reichert-Jung Scientific Instruments, Belleville, ON, Canada) and dried, and representative sections were then routinely stained with Gill’s hematoxylin and eosin (H&E). For the histochemical evaluation of gut mucins, other representative sections were stained with 1% Alcian blue (AB), pH 2.5, for 1 h (AB 2.5) for the demonstration of all acidic mucins, comprising both sialomucins (sialated or carboxylated) and/or sulfomucins (sulfated) mucins; 1% AB, pH 1.0, for 1 h (AB 1.0) for the selective identification of sulfomucins (8, 16); or a combination AB 2.5/periodic acid (5 min)-Schiff base (15 min) (PAS) reaction allowing unsubstituted α-glycol-rich neutral mucins and acidic mucins to be differentiated (19). Duplicate sections were stained with the PAS reaction after amylase digestion (PASa) to exclude any possible confounding effects of glycogen (18). PASa/AB 2.5-stained sections were used for histochemical analyses. All of the histochemical staining procedures were followed by H&E counterstaining, allowing the differentiation of mucin-secreting cells (goblet cells) from other cellular components of the gut or colonic mucosa (22). To ensure comparability between the different groups of animals, sections from all experimental groups were stained in a single batch. The histochemical staining results were interpreted as follows: 1) with PASa/AB 2.5, neutral mucins were stained red, acidic mucins were stained blue, and a purple color represented both neutral and acidic mucins present within the same goblet cell; 2) AB 2.5 stained all acidic mucins (sialomucins and sulfomucins) blue; and 3) AB 1.0 stained sulfomucins blue.

Histochemical, light microscopic, and histomorphometric analyses of stained sections were performed by an experienced certified pathologist (A. Adjiri-Awere) using a binocular light microscope at $\times 10$ ocular magnification with a $\times 10$ objective. For histochemical analyses, semiquantitative staining intensities (regardless of color) were subjectively evaluated using a scale ranging from 0 (unreactive) to 3 (intensely stained). In addition, cells in the intestinal mucosa stained with AB 1.0, AB 2.5, or PASa/AB 2.5 were counted in 10 well-oriented gut crypt-villus or colon gland-ridge units in each animal. Counts for 10 crypt-villus (or gland-ridge units) from the base of the crypt (or gland) to the tip of the villus (or edge of the glandular ridge) were pooled and expressed as means due to variations in villus or gland lengths and orientation. As a means of understanding the effects of treatment on mucin production, the product of staining intensity and the number of goblet cells observed was calculated to give an estimate of total stain (see Table 5).

Histomorphometric analyses were performed on H&E-stained tissue sections. The parameters measured were as follows: villus height ($h$; measured from the tip of the villus to the villus-crypt junction), crypt depth ($d$; measured from the crypt-villus junction to the base of the crypt), villus width at midvillus height ($mh$), and villus surface area [calculated as VSA = $\pi \times mh \times h + \pi \times mh/2$]. From each tissue section, 10 vertically oriented crypt-villus units (small intestine) and 10 colon gland-ridge units were selected, if elongated, straight, possessed a lumen that opened to the mucosal surface at the luminal margin, and had crypt or glandular base in contact with the muscularis mucosae.

**Statistical analyses.** Statistical comparisons of measured parameters between treatment groups were performed by ANOVA followed by the least-significant difference multiple-comparison test (SAS version 6.07, Cary, NC). Differences were considered to be significant if $P < 0.05$.

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**RESULTS**

**Daily weight gain.** During the course of the study, piglets in the IG-A and IV-A treatment groups were healthy and active, whereas piglets in the IG-D group became listless after 1–2 days. Initial weights (1.84 ± 0.37 kg, $n = 21$), final weights (2.73 ± 0.45 kg, $n = 21$), and daily gains (0.11 ± 0.03 kg, $n = 21$) were not different among treatment groups.

**Nitrogen retention and plasma analyses.** Nitrogen intake over the course of the study did not differ among the treatment groups ($P > 0.10$; Table 1). From day 4 to day 8 of the study, nitrogen excretion and plasma urea were higher and nitrogen retention was lower in the IG-D group compared with the IG-A and IV-A groups ($P < 0.05$; Table 1). Plasma threonine concentrations were higher in IV-A pigs versus those in IG-A and IG-D pigs (Table 1); due to the high pooled SDs, plasma threonine in IG-D pigs (44 μmol/l) was not different than that in IG-A pigs (183 μmol/l).

**Incidence of diarrhea.** Mean daily scores for all IG-D pigs over the entire study period was 2.1, which was significantly greater ($P < 0.0001$) than that for IG-A (0.06) or IV-A (0.25) pigs (Fig. 1). All six IG-D piglets (in which scores were measured) exhibited diarrhea on 35 of a possible 48 pig days, with an average severity of 2.82 over the diarrhea days. The change to severe diarrhea was abrupt by day 4 of the study. In the IG-A group, only one piglet exhibited diarrhea over a total of 2 days (of a possible 48 pig days), with an average severity of 1.5. In the IV-A group, two piglets were observed to have slight and moderate diarrhea, over 2 and 7 days (1.3 score).

**Tissue weights.** Mucosal weights were not different across treatments in the duodenum (IG-A, 91 mg/cm; IG-D, 57 mg/cm; IV-A, 80 mg/cm; pooled SD, 29), proximal jejunum (IG-A, 103 mg/cm; IG-D, 82 mg/cm; IV-A, 98 mg/cm; pooled SD, 21), midjejunum (IG-A, 106 mg/cm; IG-D, 100 mg/cm; IV-A, 104 mg/cm; pooled SD, 28), distal jejunum (IG-A, 106 mg/cm; IG-D, 105 mg/cm; IV-A, 107 mg/cm; pooled SD, 31), and ileum (IG-A, 111 mg/cm; IG-D, 88 mg/cm; IV-A, 112 mg/cm; pooled SD, 28). Relative small intestinal lengths were not different among groups (IG-A, 211 cm/kg; IG-D, 194 cm/kg; IV-A, 215 cm/kg; pooled SD, 21).

In the large intestine, IG-D piglets had significantly lower amounts of mucosa and luminal contents per centimeter in both sections (Table 2); in addition, relative lengths of the large intestine were lower in IG-D piglets.

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Table 1. Nitrogen retention and plasma urea and threonine concentrations in IG-A, IG-D, and IV-A piglets

<table>
<thead>
<tr>
<th></th>
<th>Nitrogen Intake, g kg$^{-1}$ day$^{-1}$</th>
<th>Nitrogen Output, g kg$^{-1}$ day$^{-1}$</th>
<th>Nitrogen Retention, %</th>
<th>Plasma Urea, mmol/l</th>
<th>Plasma Threonine, μmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>IG-A</td>
<td>1.97</td>
<td>0.30</td>
<td>84%</td>
<td>2.00</td>
<td>183</td>
</tr>
<tr>
<td>IG-D</td>
<td>2</td>
<td>0.71</td>
<td>66%</td>
<td>7.82</td>
<td>44</td>
</tr>
<tr>
<td>IV-A</td>
<td>2.09</td>
<td>0.30</td>
<td>84%</td>
<td>2.49</td>
<td>572</td>
</tr>
<tr>
<td>Pooled SD</td>
<td>0.45</td>
<td>0.13</td>
<td>7</td>
<td>1.42</td>
<td>115</td>
</tr>
</tbody>
</table>

Values are means; $n = 7$ piglets/group. Piglets were fed a threonine-adequate diet intragastrically (IG-A), a threonine-deficient diet intragastrically (IG-D), or a threonine-deficient diet intragastrically with adequate threonine intravenously (IV-A). Plasma urea and plasma threonine concentration data within piglets were averaged from day 4 to day 8. **For data with letter superscripts within a row, those not sharing a letter are different ($P < 0.05$, least-significant difference comparisons).**
Dietary Threonine and Gut Mucin Synthesis

**Fig. 1.** Diarrhea scores of piglets fed a threonine-adequate diet intragastrically (IG-A), a threonine-deficient diet intragastrically (IG-D), or a threonine-deficient diet intragastrically with adequate threonine intravenously (IV-A). The incidence and severity of diarrhea were assessed using a scale of 0–3: 0, no diarrhea; 1, slight diarrhea; 2, moderate diarrhea; and 3, severe, highly fluid diarrhea. Daily scores for each pig were averaged over 8 days; lines represent mean daily scores for groups. Diarrhea scores were taken for 6 of 7 piglets in each treatment group.

**Histomorphometry.** The morphology of goblet cells was typical: basally compressed nuclei and abundant apical cytoplasm. In the midjejunum and ileum, villus heights and villus height-to-crypt depth ratios were lower in IG-D pigs (Table 3). VSAs (estimated as a cylinder with a diameter equaling the villus width at the midvillus height) were lower in both IG-D and IV-A duodena. In the colon, there were no differences in crypt depth (data not shown).

**Carbohydrate analysis for mucin quantification.** As our elemental diets contained only glucose as a source of carbohydrates, any glucosamine and galactosamine measured by this procedure was assumed to be from endogenous production. Native, undigested mucin was estimated as a measure of total glycosylated mucin production, and pronase-digested mucin was estimated as a measure of unglycosylated mucin. Both native and pronase-resistant total mucin were significantly lower in the duodenum and colon of IG-D pigs versus both of the other groups (Table 4). The vast majority of native mucin was resistant to pronase digestion.

**Table 2. Large intestinal parameters in IG-A, IG-D, and IV-A piglets**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IG-A</th>
<th>IG-D</th>
<th>IV-A</th>
<th>Pooled SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length, cm/kg body weight</td>
<td>44.0 ± 3.4</td>
<td>34.3 ± 3.3</td>
<td>48.1 ± 3.8</td>
<td>9.6</td>
</tr>
<tr>
<td>Proximal colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa, mg/cm</td>
<td>64 ± 6.9</td>
<td>39 ± 6.9</td>
<td>59 ± 6.9</td>
<td>16</td>
</tr>
<tr>
<td>Muscularis, mg/cm</td>
<td>206 ± 15</td>
<td>157 ± 15</td>
<td>192 ± 15</td>
<td>60</td>
</tr>
<tr>
<td>Luminal contents, mg/cm</td>
<td>129 ± 12</td>
<td>25 ± 12</td>
<td>120 ± 12</td>
<td>67</td>
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<tr>
<td>Distal colon</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa, mg/cm</td>
<td>63 ± 6.3</td>
<td>37 ± 6.3</td>
<td>46 ± 6.3</td>
<td>21</td>
</tr>
<tr>
<td>Muscularis, mg/cm</td>
<td>143 ± 14</td>
<td>124 ± 14</td>
<td>143 ± 14</td>
<td>43</td>
</tr>
<tr>
<td>Luminal contents, mg/cm</td>
<td>101 ± 10</td>
<td>12 ± 10</td>
<td>65 ± 10</td>
<td>51</td>
</tr>
</tbody>
</table>

Values are means; n = 7 piglets/group. a,b For data with letter superscripts within a row, those not sharing a letter are different (P < 0.05, least-significant difference comparisons).
served only in the duodenum of the IG-A group versus both of
the other groups (Table 5). In almost all sections of the gut
under all treatments, stained goblet cells were predominantly
purple, although some cells also exhibited discrete dual stain-
ing with red and purple (neutral and mixed) or red and blue
(neutral and acidic) areas. The distribution of goblet cells with
different staining is shown in Fig. 2. Numbers of goblet cells
that contained neutral mucins (neutral and neutral/homoge-
neous) were not affected by threonine deficiency in any sec-
tions; however, intravenous threonine did result in more neu-
tral mucin-containing goblet cells in the ileum (Fig. 2).

Histologically, mucin-containing goblet cells in all of the small
intestinal sections of the IG-A and IV-A groups appeared more
voluminous and had a greater preponderance of purple staining
than those in the IG-D group (see the duodenum, Fig. 3). These
observations suggested that, regardless of the route of delivery
(intragastric or intravenous), adequate threonine intake supported
the production of mixtures of neutral and acidic mucins in the
small intestine.

Purple mixed mucin-containing goblet cells in all of the small
intestinal sections of the IG-A and IV-A groups were seen mainly
in the upper crypt and lower villus areas of the crypt-villus unit,
whereas goblet cells of the IG-D group were uniformly and
randomly distributed along the villus-crypt axis (see the duode-
num, Fig. 3). Blue-stained cells were few and localized to the
lower and deeper crypts of the mucosa (Fig. 3). Despite these

differences, overall PASa/AB 2.5 stain intensities (regardless of
color) were not different in any of the sections (Table 5).

In the colon, there was a distinct increase in the numbers of
acidic mucin-stained goblet cells when threonine was deficient
(Fig. 2). In the IG-D group, mucin-producing cells within
mucostral glands of the colon were smaller and less numerous
than in the IG -A group, indicating a diminished production of
mucin with threonine deficiency. Although most goblet cells
stained purple (mixed mucins) in IG-A pigs, red-stained (neu-
tral) cells were located in the lower crypts, and there were no
blue-stained (acidic) cells in the deep crypts (Fig. 3).

### Table 5. Goblet cell parameters in IG-A, IG-D, and IV-A piglets

<table>
<thead>
<tr>
<th></th>
<th>Number of Cells</th>
<th>Total Stain</th>
<th>Number of Cells</th>
<th>Total Stain</th>
<th>Number of Cells</th>
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<td></td>
<td>PAS/AB 2.5</td>
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<td>AB 1.0</td>
<td>AB 1.0</td>
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<td></td>
</tr>
<tr>
<td>Duodenum</td>
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<tr>
<td>IG-A</td>
<td>18.8 a</td>
<td>39</td>
<td>17.6 a</td>
<td>44 a</td>
<td>16.3 a</td>
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<td>15.2 b</td>
<td>41</td>
<td>5.5 b</td>
<td>6 b</td>
<td>7.1 b</td>
<td>12</td>
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<tr>
<td>IV-A</td>
<td>14.9 b</td>
<td>39</td>
<td>12.6 b</td>
<td>25 b</td>
<td>13.5 b b</td>
<td>31</td>
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<tr>
<td>Pooled SD</td>
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<td>5 c</td>
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<td>5</td>
</tr>
<tr>
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<td>20.8</td>
<td>63</td>
<td>8.7</td>
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<td>79</td>
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<td>63 a</td>
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<td>82</td>
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Values are means; n = 7 piglets/group. PAS/AB 2.5, combination of Alcian blue (AB)/periodic acid (5 min)-Schiff base (15 min) (PAS) allowing unsubstituted o-glycol-rich neutral mucins (red) and acidic mucins (blue) to be differentiated; AB 2.5, 1% AB at pH 2.5 for 1 h for the localization of sialated and/or sulfated acidic mucins; AB 1.0, 1% AB at pH 1.0 for 1 h for the selective identification of sulfomucins. Numbers of cells were counted in 10 well-oriented crypt-villus units of ~25 μm in each animal. Total stain refers to semiquantitative staining intensities based on a scale ranging from 0 (unreactive) to 3 (intensely stained) multiplied by the total numbers of goblet cells. a,b,c For data with letter superscripts within a row, those not sharing a letter are different (P < 0.05, least-significant difference comparisons).
contrast, in the IG-D and IV-A groups, acidic mucins were mostly located in the deep crypts, whereas red-stained neutral mucins were found in the zone of proliferation. AB 2.5 staining was used to indicate only acidic mucins: sialomucins and/or sulfomucins. In the duodenum and ileum, the IG-A group had a greater number of acidic mucin-producing goblet cells and total stain compared with IG-D pigs, with IV-A pigs intermediate ($P < 0.05$; Table 5). In the IG-A and IV-A groups, the pattern of staining was similar, with a decrease in staining intensity from the deep crypt to villus tip; in contrast, staining intensities were equal between the villus and crypt in IG-D pigs.

In the colon of the IG-D group, acidic mucin-stained cells were clustered in the deep glandular crypts and zone of proliferation, whereas cells of the other groups were distributed along the entire length of the colonic glands. These observations were consistent with the results of the PASa/AB 2.5 stain. Additionally, stained cells in the IG-A group were visually larger than cells from the IG-D and IV-A groups. Despite the difference in the pattern of distribution, total goblet cell num-

Fig. 3. Light micrographs of sections of the duodenal mucosa in IG-A (top), IG-D (middle), and IV-A (bottom) piglets. IG-A and IV-A micrographs show a preponderance of apparently more voluminous goblet cells, containing a mainly homogeneous mixture (purple) of both neutral and acidic mucins within each goblet cell and present mainly in the upper crypt and lower villus areas of the crypt-villus unit in the IG-A and IV-A groups, respectively. The IG-D micrograph shows a paucity of goblet cells, which were relatively less voluminous and containing a homogeneous mix of neutral and acidic mucins (purple), acidic mucins (blue), and neutral mucins (red); these goblet cells are uniformly and randomly distributed along the crypt-villus axis. AB (pH 2.5)/PAS reaction with amylase digestion, with H&E counterstain, was used. Magnification: ×100.

Fig. 4. Light micrographs of sections of the proximal colonic mucosa in IG-A (top), IG-D (middle), and IV-A (bottom) piglets. The IG-A micrograph shows a preponderance of relatively larger surface and crypt glandular goblet cells containing neutral mucins (red) as well as some with mixed mucins (neutral and acidic, purple). The IG-D micrograph shows mostly smaller goblet cells containing acidic mucins (blue), and the IV-A micrograph shows mostly intermediate-sized surface and crypt glandular goblet cells containing neutral (red) and mixed (purple) mucins, with a few small blue (acidic) goblet cells localized to the crypts. AB (pH 2.5)/PAS reaction with amylase digestion, with H&E counterstain, was used. Magnification: ×100.
bers and total staining of acidic mucin-producing cells of colonic glands were not different among groups (Table 5). These observations suggested that, regardless of the route of delivery, adequate threonine intake supported the production of sialomucins and/or sulfomucins in the small and large intestines; however, intragastric threonine appeared to be superior in some respects.

**AB 1.0 staining of sulfated acidic mucins.** Treatment with AB 1.0 stain was used to indicate only sulfated acidic mucins. In the duodenum and ileum of the IG-D group, fewer ($P < 0.05$) sulfomucin-producing goblet cells were counted than in IG-A pigs, with IV-A pigs intermediate (Table 5). In small intestinal sections of IG-A and IV-A pigs, stained cells were distributed throughout the length of the villi, whereas in IG-D pigs, sulfomucin-producing cells were primarily located in the deep crypts and midcrypts. In the colon, only total stain was significantly highest in the IV-A pigs ($P < 0.05$; Table 5).

**DISCUSSION**

Not only is threonine an essential amino acid for growth in young pigs, but its preferential utilization by the gut for mucus synthesis makes it disproportionately essential for maintenance. Because up to 90% of dietary threonine is extracted by the portal-drained viscera (versus only about a third for other essential amino acids) (24, 27, 29, 30), we hypothesized that threonine’s availability for maintenance functions in the gut would be particularly sensitive to threonine supply. The protective mucus layer in the gut predominantly consists of mucins, glycoproteins that are particularly rich in threonine. Moreover, mucins are continuously synthesized and very resistant to small intestinal proteolysis and hence recycling; therefore, mucin synthesis is largely an irreversible “loss” of threonine (29). As a result, a substantial and constant supply of threonine is necessary to maintain gut function and structure. This study clearly demonstrated that the intestinal tract is indeed very sensitive to the total dietary threonine supply as well as the route of delivery. Not only is the quantity of mucins altered when threonine is deficient, but the characteristics of the mucin carbohydrate moiety are affected, which can have significant functional outcomes. This latter effect on mucin structure is particularly striking when only threonine is provided intravenously in pigs fed otherwise complete enteral nutrition.

The neonatal piglet is very sensitive to threonine deficiency, as demonstrated by the nitrogen balance and plasma metabolite data. With dietary threonine reduced to $\sim 20\%$ of the requirement recommended by the National Research Council (21), IG-D piglets experienced a fourfold increase in plasma urea concentrations, which corresponded with a doubling of urinary nitrogen excretion and much lower nitrogen retention (Table 1). Therefore, at the whole body level, threonine limited protein synthesis, which resulted in catabolism of other amino acids. Parenteral threonine delivery did not affect any of these nitrogen metabolism parameters, most likely because the complete diet (other than threonine) was delivered gastrically. As a result, gut atrophy was not a consequence in IV-A piglets as it is when complete diets are fed parenterally, which leads to lower nitrogen retention (4). Intraosmotic threonine did, however, lead to higher plasma threonine concentrations. The lower plasma concentrations of threonine in IG-A and IG-D groups suggests that a large amount of orally administered threonine is retained or catabolized by the gut (24, 27, 29). Similarly, the high plasma concentrations in IV-A piglets suggests that threonine administered intravenously may not be efficiently utilized by the gut for mucosal protein synthesis.

Our main hypothesis was that dietary threonine restriction would reduce mucin synthesis simply by limiting the availability of the primary essential amino acid. As a direct quantitative measure of mucin quantity in the gut, mucosal scrapings from each section were analyzed according to the alditol acetates of the carbohydrate sidechains glucosamine and galactosamine. Although this approach does not quantify total mucin secretion over time, mucin quantity per length of the gut, as we measured, should reflect total mucin secretion in response to respective diets because all diets were fed continuously over 24 h for 8 days. The severely reduced mucin concentrations in the duodenum and colons of threonine-deficient piglets (with similar trends in jejunum and ilea) suggest that dietary threonine can directly affect mucin production. This decrease in mucosal mucin content could be a result of diminished mucin secretion from goblet cells or compromised de novo synthesis of mucin. The latter is more likely given the recent data in rats showing that fractional synthesis rates of mucin (but not total protein) were lower after 14 days of feeding threonine-deficient diets (13). It is also important to note that because we used elemental diets, mucin synthesis and secretion in our pigs were at a basal rate, considering that luminal bulk has been shown to stimulate mucin secretion (28). However, the lack of fibrous bulk is more physiological in our neonatal piglets, which would normally be suckling highly digestible sow milk at this stage of development.

Although crude mucin analysis can indicate gross deficiencies in mucin quantity, a more detailed description of mucin oligosaccharide properties may indicate changes in the functional characteristics of the mucus lining. The carbohydrate structures found on mucin macromolecules are extraordinarily diverse, providing a vast array of potential binding sites for both commensal and pathogenic organisms. Whether a harmful or beneficial outcome results from the attachment of microbes to intestinal mucins depends on factors such as the composition and quantity of mucins, intestinal motility, and rate of intestinal fluid flow (14). For example, it has been suggested that acidic mucins (oligosaccharide chains terminated with sialic acid or sulfate groups) protect against microbial penetration and translocation because these mucins are more resistant to bacterial mucolytic activities (12). Moreover, mucins in the neonatal piglet are particularly highly acidic, probably because the neonate’s acquired immune system is not fully functional in the intestine and the neonate is dependent on the innate defenses of mucus (9). Indeed, increased production of sulfated mucins, particularly between the midcrypt and villus tip, may be associated with the maturation of goblet cells in neonatal piglets (7). In our study, threonine-deficient piglets had dramatically lower numbers of goblet cells staining for total acidic and sulfated mucins in the duodenum and ileum (Table 5). These data suggested that IG-D piglets may have compromised innate defenses in their gut, and so it is perhaps not surprising that IG-D piglets experienced consistent, relatively severe diarrhea throughout the study. It is unclear whether some of the histological findings are a result of threonine deficiency directly or of diarrhea indirectly. Presumably, many of the main findings must be present before diarrhea as only threonine-
deficient pigs universally exhibited the onset of severe diarrhea.

The sudden onset of diarrhea is a very significant clinical outcome and is likely a result of an opportunistic infection by resident microflora. Considering the gut is positioned as the first line of defense for the body, a compromised gut barrier function of its mucus layer would leave IG-D piglets functionally immunocompromised against enteral bacteria. The carbohydrate structures of mucus are extraordinarily diverse and provide a wide array of binding opportunities for commensal and pathogenic microbes. Through these binding interactions, the mucin layer prevents microbial access to the epithelium, where full-blown inflammation would occur. The quality and quantity of this layer were obviously compromised in the threonine-deficient pigs, and this may have resulted in severe diarrhea. It is also possible that the expression of mucin subtypes was altered. The most abundant mucin synthesized in goblet cells of the small and large intestines is MUC2 (10). MUC2 is a secreted mucin that is particularly rich in threonine and is involved in forming the extracellular mucus matrix. It is reasonable to hypothesize that MUC2 expression might be disproportionately downregulated with threonine deficiency, and the resulting altered mucin subtype profile may also be partly responsible for the changes in carbohydrate profile and for the compromised barrier function. However, the porcine equivalents of these human genes have yet to be elucidated fully. Nevertheless, more research into the effects of diet on mucin subtypes is warranted. Diarrhea is an important clinical observation suggesting that the primary site of compromise during threonine deficiency is gut function. With insufficient threonine and subsequent diarrhea, not only is the neonate vulnerable to bacterial infection and fluid loss but it is also susceptible to other problems associated with malnutrition.

In the colon, although IG-D piglets had similar total goblet cell numbers as control piglets, goblet cells were smaller, and there was a distinct shift toward more acidic mucin production in these smaller cells (Figs. 2 and 4). There is a direct relationship between microbiota density and abundance of acidic mucin subtypes in the different sections of the gut (12). It is believed that an increase in the density of mucus-hydrating bacteria will lead to an increase in acidomucin synthesis and secretion (12). In our pigs, because total colonic mucin content was lower in threonine-deficient piglets, it is possible that an increase in mucolytic bacteria population reduced total secreted mucin (by digesting mucin and by causing diarrhea) despite the host’s attempt to compensate by increasing its population of acidomucin-producing goblet cells. Indeed, there were distinct large clusters of acidomucin-stained cells in the deep crypts of the colon in IG-D piglets compared with IG-A piglets, whose colonic deep crypts only stained for neutral mucins. This attempt to reconstitute the mucus layer was futile given the lack of precursors (namely, threonine) required to maintain adequate mucin synthesis rates in these cells.

Parenteral threonine supply seemed to support a relatively normal mucin staining profile. Although intravenous threonine did lead to less goblet cell staining for acidic mucins in the duodenum and greater total stain for sulfomucins in the colon, most outcome parameters were not significantly different from data in IG-A pigs, including crude mucin concentrations throughout the gut. Our histopathological analyses suggested that in the small intestine, regardless of the oral or intravenous route of delivery, adequate threonine intake preferentially supported the production of goblet cells containing mixed (neutral and acidic) mucins over those containing acidic or neutral mucins alone. This preference was particularly evident in the duodenum, with the parameters being slightly superior in the IG-A than IV-A piglets (Fig. 3). In the colon, adequate levels of orally supplied threonine preferentially supported the production of goblet cells containing neutral mucins (and some with mixed mucins) over those containing acidic mucins alone (Fig. 4). In contrast, intravenously supplied threonine supported more goblet cells containing only acidic mucins as opposed to neutral and mixed mucins. In addition, colonic goblet cells were generally smaller with intravenous threonine than with intragastric threonine. Despite these distinct qualitative changes, most quantitative data suggested parenteral threonine was utilized effectively. Whether a longer adaptation to parenteral threonine infusion would have further compromised gut function is unknown.

Recently, Schaart et al. (24) demonstrated that dietary, as opposed to arterial, threonine was preferentially utilized by the portal-drained viscera in orally fed pigs with a particular sequestration of label in the small intestine. Such data would suggest that in our IV-A piglets, mucin synthesis should have been significantly compromised when threonine was supplied intravenously. However, although some changes were observed in our study, it is possible that 8 days of adaptation to our regimen allowed IV-A pigs to upregulate arterial uptake of threonine in the face of a dietary threonine deficiency. Plasma threonine concentrations were threefold higher in IV-A piglets, so infused threonine was available to the gut. In addition, total parenteral nutrition (TPN) is known to atrophy gut mucosa, compromise gut barrier function, and dramatically alter mucin characteristics (11). Because our IV-A piglets were fed total enteral nutrition (except for threonine), our data suggest that changes due to TPN feeding are not specifically a result of unavailability of arterial threonine. On the other hand, the expansion of acidomucin goblet cell populations observed during TPN (11) is consistent with our data in the colons of threonine-deficient piglets, which may suggest that they both share a common defense process to avoid gut barrier failure.

Similar to the effects of TPN feeding (4), gut morphological parameters were compromised by oral feeding of threonine-deficient diets. Although threonine deficiency had no effect on small intestinal mucus mass, IG-D piglets did experience a significant decrease in villus height throughout the small intestine, which is often suggested to indicate compromised enterocyte differentiation and function (Table 3). In addition, the overall length and mucus mass throughout the colon were lower when threonine was unavailable (Table 2). Because these piglets were growing rapidly, in effect, low dietary threonine was limiting large intestinal growth in IG-D piglets. There was also a significantly lower mass of luminal contents in the large intestine of IG-D pigs. Because we used elemental diets, luminal contents did not contain any significant amount of dietary matter and would reflect mucus and microflora mass. Although this crude measure is consistent with the lower mucin content measured in mucosal scrapings of the colon in threonine-deficient piglets, it is important to note that these pigs also had diarrhea, which would contribute to the emptying of colonic contents.
The importance of threonine in the neonatal piglet cannot be overstated and has been well demonstrated by this study. Piglets receiving intragastric nutrition require roughly twice the amount of threonine as piglets fed by intravenous infusion (3). The difference in requirement is likely due to the role of threonine in the piglet gut for intestinal mucin synthesis. Piglets receiving diets deficient in threonine alone showed symptoms of diarrhea, increased plasma urea, and decreased symptoms of diarrhea and normal plasma urea and mucosal weight. While these neonatal piglets were able to utilize threonine supplied either orally or intravenously, there was some evidence that the oral route was preferred. Given the importance of threonine in the structure and function of the gastrointestinal tract, and that the rates of mucin synthesis and secretion are likely high, the dietary requirement of threonine is likely affected by changes in the gut, such as during gut atrophy and regrowth. Oral threonine during refeeding may be very beneficial for the return of “normal” gut function.

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GRANTS

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REFERENCES