Carbohydrate maldigestion induces necrotizing enterocolitis in preterm pigs

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Thymann T, Møller HK, Stoll B, Støy AC, Buddington RK, Bering SB, Jensen BB, Olutoye OO, Siggers RH, Mølbak L, Sangild PT, Burrin DG. Carbohydrate maldigestion induces necrotizing enterocolitis in preterm pigs. Am J Physiol Gastrointest Liver Physiol 297: G1115–G1125, 2009. First published October 1, 2009; doi:10.1152/ajpgi.00261.2009.—Necrotizing enterocolitis (NEC) remains the most serious gastrointestinal disorder in preterm infants. It is associated with the initiation of enteral nutrition and may be related to immature carbohydrate digestive capacity. We tested the hypothesis that a formula containing maltodextrin vs. a formula containing lactose as the principal source of carbohydrate would predispose preterm pigs to a higher NEC incidence. Cesarean-derived preterm pigs were given total parenteral nutrition for 48 h followed by total enteral nutrition with a lactose-based (n = 11) or maltodextrin-based (n = 11) formula for 36 h. A higher incidence (91% vs. 27%) and severity (score of 3.3 vs. 1.8) of NEC were observed in the maltodextrin than in the lactose group. This higher incidence of NEC in the maltodextrin group was associated with significantly lower activities of lactase, maltase, and aminopeptidase; reduced villus height; transiently reduced in vivo aldohexose uptake; and reduced ex vivo aldohexose uptake capacity in the middle region of the small intestine. Bacterial diversity was low for both diets, but alterations in bacterial composition and luminal concentrations of short-chain fatty acids were observed in the maltodextrin group. In a second study, we quantified net portal absorption of aldohexoses (glucose and galactose) during acute jejunal infusion of a maltodextrin- or a lactose-based formula (n = 8) into preterm pigs. We found lower net portal aldohexose absorption (4% vs. 42%) and greater intestinal recovery of undigested carbohydrate (68% vs. 27%) in pigs acutely perfused with the maltodextrin-based formula than those perfused with the lactose-based formula. The higher digestibility of the lactose than the maltodextrin in the formulas can be attributed to a 5- to 20-fold higher hydrolytic activity of tissue-specific lactase than maltases. We conclude that carbohydrate maldigestion is sufficient to increase the incidence and severity of NEC in preterm pigs.

lactose; maltodextrin; premature; enteral nutrition

NECROTIZING ENTEROCOLITIS (NEC) remains the most serious gastrointestinal (GI) disease in preterm infants, yet its pathogenesis is poorly understood. Epidemiological evidence has established that prematurity is the most significant risk factor for development of NEC (31). In premature infants, the onset of enteral feeding and bacterial colonization are two additional factors that present early after birth to the developing gut and have been linked to NEC (12, 27, 33). The etiology of enteral feeding as a causal factor in NEC has been ascribed to insufficient digestive capacity of the premature intestine. This has especially been the case for the carbohydrate lactose as a result of the late-gestational increase in lactase activity compared with the earlier-gestational increases in maltase-glucocylamylase and sucrase-isomaltase activities (22, 23). In addition, introduction of enteral nutrition is often delayed in preterm infants because of their immature gastromotility and digestive functions. Instead, they receive parenteral nutrition, which may further delay or reduce the maturation of digestive enzymes. Studies in premature infants demonstrate that lactose digestion and glucose absorption increase with gestational age but that some degree of lactose malabsorption and colonic fermentation still occurs (23, 43, 45, 46). Although colonic fermentation of lactose is accompanied by an increase in gas production, there is no clear evidence that this phenomenon induces NEC in infants (23). The direct link between lactose malabsorption and NEC has not been proven. Yet commercial preterm formulas contain a mixture of lactose and glucose polymers to reduce the dietary lactose load. The glucose polymer mixture used in preterm formulas consists of corn syrup solids derived from partially hydrolyzed cornstarch. These glucose polymers are considered safe and are well tolerated in preterm formulas. However, the relationship between digestion of these dietary carbohydrates and NEC in animals or infants has not been directly examined.

The key role of bacteria in NEC is based on clinical evidence of luminal bowel gas production (e.g., pneumatosis intestinalis), increased products of bacterial fermentation [e.g., shortchain fatty acids (SCFA)], and epidemic outbreaks of NEC. The most compelling experimental evidence for the role of bacteria in development of NEC was first reported from studies in the germ-free quail. This species is lactase deficient, and colonization with infant fecal bacteria induces NEC when the birds are fed a lactose-based formula (10, 50, 52). The quail studies imply that maldigestion and bacterial fermentation of dietary lactose trigger NEC. These studies also implicated specific bacterial species such as Clostridia in the etiology of NEC, and these bacteria have also been reported in several cases of human NEC (16). More recently, we showed that elimination of the microbiota by rearing formula-fed, preterm pigs in a germ-free isolator or by feeding formula to fetal pigs in utero prevents the development of NEC that normally occurs...
in conventionally reared newborn preterm pigs (4, 5, 41). Thus, although bacterial colonization seems necessary for NEC to occur, it is unclear whether carbohydrate maldigestion is a necessary or permissive GI process to facilitate activation of the mucosal inflammatory response by luminal bacteria and their toxins.

The aim of the present study was to directly test whether the nature of the dietary carbohydrate source influences the development of NEC in preterm pigs. We hypothesized that feeding a diet containing maltodextrin, rather than lactose, as the primary source of dietary carbohydrate would predispose preterm pigs to NEC. This is based on the evidence suggesting that the newborn pig intestinal enzyme capacity is better suited to hydrolysis of lactose than maltodextrin (2, 32). We compared diets that were formulated to contain predominantly lactose or glucose polymers (maltodextrin) as the carbohydrate source while maintaining all other macronutrients the same. The incidence and severity of NEC with the different diets were compared, and we examined whether this correlated with other elements of carbohydrate digestion, aldohexose absorption, and microbial colonization. Finally, we investigated the aldohexose absorptive capacity of the preterm pig intestine by acutely infusing the two different formulas into the jejunum of anesthetized pigs.

MATERIALS AND METHODS

Experiment 1

Experimental design and diets. Twenty-two preterm pigs were delivered by cesarean section from two sows (Large White × Danish Landrace × Duroc; Askelygaard, Roskilde, Denmark) at 105 days (92%) gestation, as described previously (40). The pigs were immediately transferred to heated (37°C) and oxygenated incubators, an anesthetized pigs.

Twenty-two preterm pigs were compared, and we examined whether this correlated with other elements of carbohydrate digestion, aldohexose absorption, and microbial colonization. Finally, we investigated the aldohexose absorptive capacity of the preterm pig intestine by acutely infusing the two different formulas into the jejunum of anesthetized pigs.

Postmortem sample collection. Pigs were killed when clinical symptoms of NEC (abdominal distension, lethargy, cyanosis, or bloody diarrhea) were observed or at the conclusion of the period of enteral nutrition, with anesthesia induced with butorphanol, ketamine, xylazine, zolazepam, and tiletamine, followed by a lethal intracardiac injection of pentobarbitone sodium. Immediately after the animal was killed, the abdomen was opened and filled with ice. The GI tract (GIT) was removed, and the small intestine, from the pyloric sphincter to the ileocecal junction, was isolated by cutting along the mesenteric border. The colon, stomach, and each small intestinal segment (proximal, middle, and distal) were evaluated for pathological lesions, which were scored as follows: 1, no or minimal focal hyperemia; 2, mild focal hyperemia; 3, moderate locally extensive hyperemia; 4, severe focal hyperemia; 5, severe locally extensive hemorrhage and necrosis; and 6, severe extensive hemorrhage and necrosis. A score of ≥3 in any region was indicative of NEC.

After the different regions of the GIT were isolated and NEC scores were assigned, the luminal contents from the distal small intestine were collected for analysis of the gut microbiota and for measurement of SCFA concentrations. After removal of the remaining contents, the entire small intestine was weighed and divided into proximal, middle, and distal segments of equal length. Full-thickness tissue samples were collected from all GIT sections and snap frozen in liquid nitrogen. Another set of tissue samples from the same sampling sites was fixed in 4% neutral buffered paraformaldehyde for 24 h and then transferred to 70% ethanol for histological processing and observation of tissue architecture to confirm gross observations recorded during the necropsy. A third set of samples from each of the three small intestine segments was transferred to ice-cold PBS buffer and used for measurement of ex vivo tissue uptake. Intact tissue glucose uptake was measured as described previously (7, 21). The proximal, middle, and distal segments of the small intestine were everted and mounted on steel rods of similar diameter and held in ice-cold, aerated (5% CO₂–95% O₂) mammalian Ringer solution. Areas with focal lesions were avoided, unless the entire segment was affected. Beginning 45 min after death, the tissues were incubated in Ringer solution (7 min, 37°C) and then in aerated, stirred Ringer solution (2 min, 37°C) with D-[14C]glucose (0.004 mmol/l), in the absence or presence of 50 mmol/l D-glucose, to saturate the transporters. 1-[3H]glucose was collected before and 20, 40, and 60 min after each bolus. Plasma concentrations of galactose and mannitol were measured as previously described (51).

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Table 1. Macronutrient composition of milk formulas

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<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
<td>Maltodextrin</td>
<td>Lactose</td>
</tr>
<tr>
<td>Energy, kJ/l</td>
<td>4,633.8</td>
<td>4,647.8</td>
</tr>
<tr>
<td>Protein, g/l</td>
<td>61.6</td>
<td>62.6</td>
</tr>
<tr>
<td>Total carbohydrate, g/l</td>
<td>56.2</td>
<td>57.2</td>
</tr>
<tr>
<td>Lactose, g/l</td>
<td>0</td>
<td>47.7</td>
</tr>
<tr>
<td>Maltodextrin, g/l</td>
<td>55.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Other sugars, g/l</td>
<td>5.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Fat, g/l</td>
<td>70.2</td>
<td>70.2</td>
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</table>

Diet used for experiment 1 contained Seravit, Liquigen medium-chain triglyceride, and Calogen long-chain triglyceride (Nutricia, Allerød, Denmark); Variolac and Lacreprodan alpha-15 (ARLA Foods Ingredient, Viby, Denmark), and Polycose (Abbott Nutrition, Columbus, OH). Diet used for experiment 2 contained Enfamil Lactose-Free (maltodextrin-based formula) or Enfamil with Iron (lactose-based formula; Mead Johnson, Evansville, IN).
added to the incubation solution for simultaneous correction of o-glucose adherent to the tissue and absorbed independent of transporters. After the incubation, the tissues were rinsed for 20 s in cold Ringer solution, removed from the solution, weighed, and solubilized. Scintillant (Ultima Gold, PerkinElmer) was added, and associated radioactivity was measured (Tricarb 2100TR, Packard Instruments, Meriden, CT). Calculated rates of transport were normalized to tissue mass. These values were multiplied by the weight of each intestinal region (proximal, middle, or distal), and the regional values were summed to estimate total glucose transport capacities of the entire small intestine.

Microbiota analysis. The number of Lactobacillus colony-forming units (CFU) was determined using serial dilutions of luminal contents from the distal small intestine that were plated on Man-Rogosa-Sharp selective medium-agar plates (14) and incubated in aerobic and anaerobic conditions at 37°C for 2 days. Enumeration was performed on the highest countable dilution. In addition, terminal restriction fragment (T-RF) length polymorphism (T-RFLP) analysis was used to examine tissue samples from the distal small intestine. Total DNA was extracted from tissue samples from the distal small intestine by the manufacturer DNA Mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions, with the addition of a bead-beating step. The tissue samples were suspended in 1,200 μl of AE buffer (Qiagen) and processed with zirconia-silica beads (0.1 mm; Biospec Products, Bartlesville, OK) for 3 min at high speed using a Mini Beadbeater (Biospec Products). The DNA was stored at −20°C until further analysis. T-RFLP was profiled and analyzed mainly as described previously (35). Extracted total DNA was adjusted to 5 μg/ml by spectrophotometry. The universal bacterial primers S-D-Bact-0008-a-20 (5′-AGAGTTTGATCMTGGCTCAG-3′) (29) and S-D-Bact-0926lam-a-A-20 (5′-CCGTCATTCTTTAGTTT-3′) were used to make four replicate 50-μl PCR mixtures were made from each sample (35). Primer S-D-Bact-0926lam-a-A-20 was labeled with 5′-FAM (carboxyfluorescein-N-hydroxysuccinimide ester dimethylsulfosiloxane), Purified PCR products (200 ng) were digested with 20 U of Hha I (Boehringer, Mannheim, Germany) restriction enzyme in 20-μl reaction mixtures for 3 h at 37°C. The four PCR replicates were pooled into two samples, which were analyzed by electrophoresis on an automatic sequence analyzer (ABI Prism 373, PE Biosystems, Foster City, CA). ABI traces were imported into BioNumerics version 4.5 (Applied Maths) and aligned using the internal standards. T-RFs between 35 and 625 bp observed in both PCR duplicates were included in the preceding analysis. The mean relative intensity of each T-RF within a sample was calculated by dividing the area of that individual peak by the total area of all peaks on the electropherogram. For identification of possible bacterial species corresponding to the individual T-RFs, a reference list of virtual digests was obtained by insertion of the primer sequences and restriction enzyme into the in silico MiCA III virtual digests of the 16S rRNA gene using the ribosomal database (47).

Intestinal enzyme activities. Frozen tissue samples from the proximal, middle, and distal small intestine were homogenized in 1.0% Triton X-100 (6 mg/l wet tissue). Activities of lactase (EC 3.2.1.23-62), sucrase (EC 3.2.1.48-10), maltase (EC 3.2.1.20), dipeptidylpeptidase IV (EC 3.4.14.5), aminopeptidase N (EC 3.4.11.2), and amino- peptidase A (EC 3.4.11.7) in the homogenates were determined spectrophotometrically using lactose, sucrose, maltose, glycy1-4-proline-4-nitroanilide, L-alanine-4-nitroanilide, and α-glutamic acid-4-nitroanilide, respectively, as substrates according to previous protocols (42). The amount (μmol) of substrate hydrolyzed per minute at 37°C was considered to represent one unit of enzyme activity. Enzyme activity was expressed per gram of wet intestine.

SCFA analysis. Concentrations of SCFA in the contents of the stomach and colon were measured as described previously (20). Briefly, each sample was diluted in a sodium hydroxide solution containing 2-ethylbutyric acid as the internal standard. The diluted sample was extracted with HCl and diethyl ether. After centrifugation (3,000 g, 10 min), the ether layer was isolated, added to N-methyl-N-tert-butyl(dimethyl)silyl trifluoroacetamide, and incubated at 80°C for 20 min and then at room temperature for 48 h before injection into a gas chromatograph (GC). The chromatograms were integrated using Hewlett-Packard GC ChemStation software.

**Experiment 2**

**Experimental design and diets.** Sixteen pigs were delivered by cesarean section from two sows (Large White × Hampshire × Duroc; Texas Department of Criminal Justice, Huntsville, TX) at 105 days (92%) gestation and immediately transferred to cages in a room maintained at 37°C. Pigs were anesthetized by inhalation of isoflurane (2%), and a vascular catheter (Tygon tubing, 1.77 mm OD) was inserted into the umbilical artery. Pigs also received daily intravenous injections of enrofloxacin (Baytril, 5 mg·kg⁻¹·day⁻¹). The pigs were given continuous TPN, initially at a rate of 5 ml·kg⁻¹·h⁻¹ and gradually increasing to 10 ml·kg⁻¹·h⁻¹ to maintain slightly positive energy balance. The nutrient composition of the TPN solution was identical to that described previously (9, 49). All procedures were approved by the Animal Care and Use Committee of the Baylor College of Medicine and were conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

**Intestinal infusion protocol.** After 1–3 days of TPN, pigs were anesthetized and surgically implanted with an ultrasonic flow probe (size 4S, Transonics, Ithaca, NY) around the portal vein and a catheter (polyethylene tubing) in the portal vein. A silicone rubber tube was inserted into the jejunal 20 cm distal to the ligament of Treitz. The previously implanted umbilical arterial catheter was used for sampling. Body temperature, heart rate, and blood O₂ saturation were continuously monitored and recorded throughout the surgery-and-infusion protocol. Anesthesia was maintained with isoflurane (0.5–1.0%) during the infusions.

Pigs were randomized into groups that received intrajejunal infusions of a lactose-based milk formula (Enfamil with Iron, Mead Johnson, Evansville, IN; n = 8) or a maltodextrin-based infant milk formula (Enfamil Lactose-Free, Mead Johnson; n = 8; Table 1). The calculated osmolarities of the formulas were 105 mosM (maltodextrin) and 175 mosM (lactose). The infusion protocol consisted of a baseline intrajejunal saline infusion (10 ml/kg) for 30 min followed immediately by primed-continuous infusion (priming at 10 ml/kg body wt and continuous infusion at 10 ml·kg⁻¹·h⁻¹ for 120 min) of the maltodextrin- or lactose-based formula. Carbohydrate was infused at 475 mg·kg⁻¹·h⁻¹, and protein and fat were infused at 95 and 28 mg·kg⁻¹·h⁻¹, respectively. Throughout the infusions, 200-μl blood samples from the arterial and portal catheters were collected every 10 min and analyzed immediately for hematocrit, lactate, glucose, and galactose (Yellow Springs Instrument, Yellow Springs, OH).

**Sample collection and analyses.** At the end of the infusion protocol, the pigs were killed with an overdose of isoflurane, and the small intestine was immediately removed, placed on ice, and flushed with 25 ml of cold saline. Contents of the stomach and small intestine were collected, and the volumes were recorded and snap frozen in liquid nitrogen. The small intestine was weighed and divided into proximal and distal segments of equal length. Segments from the middle of each region were collected and snap frozen in liquid nitrogen for analysis of digestive enzyme activity. The concentrations of lactose and maltodextrin in the luminal contents of the stomach and small intestine were measured after deproteinization, centrifugation, and hydrolysis of lactose by β-galactosidase (G-5635, Sigma) and maltodextrin by amyloglucosidase (A-7420, Sigma). Free galactose and glucose were measured spectrophotometrically, as described previously (51). Activities of lactase (EC 3.2.1.23-62), sucrase (EC 3.2.1.48-10), maltase (EC 3.2.1.20), dipeptidylpeptidase IV (EC 3.4.14.5), aminopeptidase N (EC 3.4.11.2), and aminopeptidase A (EC 3.4.11.7) were measured using methods described for experiment 1.
Calculations. The net portal balance (NPB) for glucose and galactose was calculated by multiplying the arteriovenous concentration difference between the umbilical artery and the portal vein by the mean rate of portal blood flow at an interval of 5 min around the time of blood sampling. NPB for glucose (from digested maltodextrin or lactose) and galactose (from digested lactose) was calculated and plotted for all pigs, and the area under the curve was calculated using Graph Pad Prism (version 4.03, Graph Pad Software).

Statistics

Differences in the incidence of NEC between maltodextrin and lactose groups were evaluated using Fisher’s exact test. Data collected over time were analyzed as repeated measures using a Gaussian model of spatial correlation in the MIXED procedure of SAS (version 9.1, SAS Institute, Cary, NC) or Graph Pad Prism. For comparison between the individual peaks in the T-RFLP analysis, a two-tailed Monte Carlo estimate from the nonparametric analysis (Mann-Whitney U-test), a Proc npar1way procedure of SAS, was used. All other statistical analyses of continuous data were based on parametric ANOVA using the MIXED model of SAS. P < 0.05 was considered significant.

RESULTS

Experiment 1

The incidence and severity of NEC were greater in the maltodextrin than the lactose group (Table 2). The higher incidence and greater severity of NEC in the maltodextrin group coincided with a decline in body weight gain (−1.2 ± 4.1 vs. +12.2 ± 3.5 g/day, P < 0.05; Table 2). The relative masses of the stomach and intestine (g/kg body wt) tended to be higher for the maltodextrin group, whereas livers tended to be larger in the lactose group (Table 1). Intergroup differences were not detected for the heart, lung, spleen, and kidneys (data not presented). We found that the villus height and area were higher in the lactose group than in the maltodextrin group in the proximal, but not distal, small intestine (Table 2, Fig. 1). We also found extensive evidence of villus erosion, pneumatisis, and inflammation in NEC pigs (Fig. 1).

Table 2. NEC incidence and organ dimensions in preterm pigs fed lactose- or maltodextrin-based formulas

<table>
<thead>
<tr>
<th></th>
<th>Lactose</th>
<th>Maltodextrin</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>NEC incidence</td>
<td>27% (3/11)</td>
<td>91% (10/11)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mean NEC severity</td>
<td>1.8±0.2</td>
<td>3.3±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight at birth, g</td>
<td>976±64</td>
<td>991±64</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight gain, g/day</td>
<td>12.2±3.5</td>
<td>-12.4±1.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Relative stomach weight, g/kg body wt</td>
<td>6.1±0.6</td>
<td>8.4±1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Relative small intestinal weight, g/kg body wt</td>
<td>27.1±1.4</td>
<td>32.9±2.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Relative liver weight, kg</td>
<td>body wt</td>
<td>31.2±1.2</td>
<td>27.4±1.6</td>
</tr>
<tr>
<td>Mucosa percentage</td>
<td>53.1±4.0</td>
<td>56.1±4.0</td>
<td>NS</td>
</tr>
<tr>
<td>Jejunum villus height, μm</td>
<td>625±60</td>
<td>434±26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Jejunum villus area, μm²</td>
<td>44,962±5,051</td>
<td>30,443±1,972</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ileum villus height, μm</td>
<td>657±66</td>
<td>612±59</td>
<td>NS</td>
</tr>
<tr>
<td>Ileum villus area, μm²</td>
<td>52,776±5,521</td>
<td>51,134±6,431</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. NEC, necrotizing enterocolitis; NS, not significant.

Enzyme activity. Activities of five of the six brush-border membrane enzymes assayed were significantly higher in the lactose than in the maltodextrin group, with the differences more pronounced in the middle and distal regions of the small intestine (Fig. 2). Only sucrase activity was higher in the maltodextrin group. Among the three disaccharidases, total intestinal activity was 5- to 6-fold higher for lactase than maltase and ~80-fold higher for lactase than sucrase, indicating that the capacity to hydrolyze lactose exceeded the capacity to hydrolyze other disaccharides. Because of our previous experience that use of maltodextrin as a substrate yields detectable measurement of maltase-glucoamylase activity, we focused strictly on maltase activity using maltose as substrate.

In vivo galactose and mannitol absorption. After 6 h of enteral nutrition, the increase in plasma galactose and mannitol following the bolus was greater for the lactose than the maltodextrin group (P < 0.01; Fig. 3, A and B). The differences between groups for galactose and mannitol absorption were not apparent after 36 h of enteral nutrition (Fig. 3, C and D).

Ex vivo tissue glucose absorption. Tissue-specific glucose transport rates were higher for the lactose than the maltodextrin group in the middle, but not the proximal or distal, region of the small intestine (Fig. 4). Similarly, the total capacities for glucose transport in the entire intestinal segment were higher only in the middle region. This is consistent with the lack of treatment differences for the in vivo galactose absorption test after 36 h of TEN and just before euthanasia and tissue collection.

Microbiology. The densities of lactobacilli (CFU/g wet wt) in luminal contents collected from the distal small intestine at the time of necropsy were similar for both groups under anaerobic (1.6 × 10^{10} and 1.7 × 10^{10} CFU/g for lactose and maltodextrin groups, respectively) and aerobic (7.5 × 10^{9} and 1.8 × 10^{10} CFU/g for lactose and maltodextrin groups) culture conditions. Culture-independent analysis of the microbiota in the distal small intestine revealed a total of 31 T-RFs with an average of 10 ± 1 for the lactose group and a total of 40 T-RFs with an average of 11 ± 1 for the maltodextrin group. The T-RFs that represented >1% of total intensity are presented in Fig. 5. Regardless of treatment, the majority of total band intensity was represented by relatively few T-RFs. This indicates that the bacterial assemblage is rather simple and dominated by a few species. The predicted identities of the dominating T-RFs were determined from the virtual digests using the RPDII database (Table 3). The intensity of the T-RF at 611 bp (predicted identity: Weisella spp.) was significantly higher in the lactose than the maltodextrin group (22% and 5% of the total microbiota, respectively, P < 0.05). In contrast, T-RF intensities at 209 and 550 bp (predicted identity: Leuconostoc spp., Pseudomonas spp., or Streptococcus spp.) were significantly lower in the lactose group (P < 0.05). Intensities of the remaining TR-F bands did not differ between the two groups.

SCFA concentrations. The dominant SCFA in the luminal contents of the stomach was octanoic acid, followed by acetic and lactic acids (Fig. 6). Although intergroup differences were not detected for any of the individual SCFA, the total concentration of SCFA in the stomach was higher for the lactodextrin group (P < 0.05). The dominant SCFA in the colon of both groups, lactic acid, was significantly higher in the lactose group (P < 0.05). Succinic acid was a significant component of the...
total concentration of SCFA in the colon of the lactose, but not the maltodextrin, group (P < 0.01).

Experiment 2

During the 120-min formula infusion period, the amount of aldohexose infused was 6.50 mmol/kg in the form of glucose in the maltodextrin group. Similarly, aldohexose (3.25 mmol/kg), each as glucose and galactose units, was infused into the lactose group. NPB showed a significantly higher glucose absorption during intestinal infusion of the lactose- than the maltodextrin-based formula (P < 0.0001; Fig. 7A, top). During infusion of the lactose-based formula, the net portal absorption rate was significantly less for galactose than for glucose, despite their 1:1 molar ratio in the lactose molecule. This was reflected in a lower cumulative portal absorption rate of galactose (0.87 mmol/kg, 27% of intake) than glucose (1.90 mmol/kg, 58% of intake, P < 0.05; Fig. 7B). As expected, galactose was not detected in the arterial or venous plasma after infusion of the maltodextrin-based formula. The cumulative portal absorption of total aldohexose (glucose and galactose, 2.77 mmol/kg) was

Fig. 1. Small intestinal morphology in jejunum from pigs fed lactose- or maltodextrin-based formula after 48 h of total parenteral nutrition. Villus height in proximal intestine was higher in pigs fed lactose-based formula (A) than in pigs fed maltodextrin-based formula (B). Additional images show evidence of mucosal villus erosion, pneumatosis, and fibrin deposition in ileum (C) and colonic submucosal inflammation (D) in sections from pigs with necrolyzing enterocolitis fed maltodextrin-based formula in experiment 1.

Fig. 2. Brush-border enzyme activity of lactase, maltase, sucrase, aminopeptidase A and N, and dipeptidylpeptidase IV in proximal, middle, and distal small intestine of preterm pigs fed lactose-based (lactose, n = 11) or maltodextrin-based (maltodextrin, n = 11) formula. Values are means ± SE. Significant differences between diet groups within each region: *P < 0.05; **P < 0.01; ***P < 0.001.
42% of the lactose infused. In contrast, the cumulative portal absorption of glucose was only 4.1% of the maltodextrin infused. In the lactose group, we recovered 14% and 27% of the lactose infused in the stomach and small intestinal luminal contents, respectively. In contrast, in the maltodextrin group, we recovered 12% and 68% of the maltodextrin infused in the stomach and small intestinal luminal contents, respectively (Fig. 7C). The amount of aldohexose recovered in the small intestinal contents was higher (68% vs. 27%) in the maltodextrin than the lactose group. Among all the fractions we measured, ~15–17% of the carbohydrate load infused in the lactose and maltodextrin groups was unaccounted for. We presume that this portion of the carbohydrate load was metabolized by the gastrointestinal tissues in the first pass or passed into the cecum during the 120-min infusion period.

**Enzyme activity.** After the intestinal infusion protocol, the activities of the three disaccharidases measured in the proximal and distal small intestine did not differ between pigs receiving the lactose-based formula and those receiving the maltodextrin-based formula. Averaged across intestinal regions, the lactase activity (40–11 and 39–5 U/g) markedly exceeded the activities of maltase (1.6–0.4 and 1.5–0.4 U/g) and sucrase (0.23–0.1 and 0.23–0.1 U/g) in the lactose and maltodextrin groups, respectively. These findings corroborate the lower recovery of glucose after infusion of maltodextrin than after infusion of lactose.
These studies represents the hydrolytic product of starch and lactose to glucose and galactose. The maltodextrin used in Lactase is the main intestinal enzyme that hydrolyzes ingested lactase than either maltase or sucrase in the prenatal pig. Enzymes in our preterm pigs confirm a much higher activity of compared with pigs fed a lactose-based formula. We also show that maldigestion of maltodextrin corresponded probably due to relatively low maltase and sucrase activities. The pig has a relatively low activity of enzymes that digest piglet to digest and tolerate maltodextrin in vivo was unknown. The pig has a relatively low activity of enzymes that digest maltodextrin compared with lactase and, thus, might be expected to have digestive problems when fed maltodextrin diets. However, we previously showed that full-term pigs have few maltodextrin polymers to free glucose is sucrase-isomaltase and, to a lesser degree, maltase. In the present study, specific activity of lactase was severalfold higher than specific activity of maltase (~10-fold) and sucrase (~40-fold). In addition, the activities of maltase, lactase, and aminopeptidases were reduced in distal intestinal segments in our maltodextrin group relative to our lactose group. On the contrary, the corresponding sucrase activity tended to be higher. The lower capacity to digest maltodextrin than lactose was also supported by the limited portal absorption of aldohexoses after infusion of the maltodextrin-based formula. Furthermore, it was supported by the greater percentage of maltodextrin remaining in the stomach and small intestine in the intestinal infusion experiment. Thus our results suggest that the premature pig intestine has a severely limited enzymatic capacity to hydrolyze maltodextrin compared with lactose.

The combined results from the in vivo and ex vivo measurements of aldohexose absorption provide insights into the spatial and temporal responses of the GI epithelium of preterm pigs fed the two formulas. After 6 h of enteral feeding, which represents only two feedings, in vivo galactose absorption was lower in the maltodextrin than the lactose group. In vivo digestion of starch and lactose exists as short-chain polymers containing ~4–20 glucose units (39). Quezada-Calvillo et al. (39) showed that the primary intestinal enzyme capable of complete hydrolysis of the maltodextrin polymers to free glucose is sucrase-isomaltase and, to a lesser degree, maltase. In the present study, specific activity of lactase was severalfold higher than specific activity of maltase (~10-fold) and sucrase (~40-fold). In addition, the activities of maltase, lactase, and aminopeptidases were reduced in distal intestinal segments in our maltodextrin group relative to our lactose group. On the contrary, the corresponding sucrase activity tended to be higher. The lower capacity to digest maltodextrin than lactose was also supported by the limited portal absorption of aldohexoses after infusion of the maltodextrin-based formula. Furthermore, it was supported by the greater percentage of maltodextrin remaining in the stomach and small intestine in the intestinal infusion experiment. Thus our results suggest that the premature pig intestine has a severely limited enzymatic capacity to hydrolyze maltodextrin compared with lactose.

The specific activities of the major intestinal hydrolase enzymes in our preterm pigs confirm a much higher activity of lactase than either maltase or sucrase in the prenatal pig. Lactase is the main intestinal enzyme that hydrolyzes ingested lactose to glucose and galactose. The maltodextrin used in these studies represents the hydrolytic product of starch and

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Table 3. Possible identity of bacterial species of dominating T-RFs

<table>
<thead>
<tr>
<th>T-RF</th>
<th>Possible Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>209</td>
<td>Leuconostoc spp., Pseudomonas spp.</td>
</tr>
<tr>
<td>218</td>
<td>Enterococcus spp.</td>
</tr>
<tr>
<td>234</td>
<td>Clostridium spp.</td>
</tr>
<tr>
<td>373</td>
<td>Enterobacteriaceae bacterium, Klebsiella, Salmonella enterica, Escherichia coli, Vibrio spp., Bifidobacterium</td>
</tr>
<tr>
<td>375</td>
<td>Vibrio australianus, Actinomyces, Salmonella enterica</td>
</tr>
<tr>
<td>497</td>
<td>Unknown</td>
</tr>
<tr>
<td>550</td>
<td>Streptococcus spp.</td>
</tr>
<tr>
<td>569</td>
<td>Vibrio spp., Oxalobacter formigenes</td>
</tr>
<tr>
<td>571</td>
<td>Salmonella spp., Vibrio spp.</td>
</tr>
<tr>
<td>583</td>
<td>Lactococcus garvieae, Streptococcus spp.</td>
</tr>
<tr>
<td>593</td>
<td>Streptococcus spp., Capnocytophaga ochracea, Dialister, Megaspheera elsdeni, Peptococcaceae bacterium</td>
</tr>
<tr>
<td>597</td>
<td>Lactobacillus rhamnosus, Lactobacillus brevis, Lactobacillus acidophilus</td>
</tr>
<tr>
<td>611</td>
<td>Weissella cibaria, Weissella halotolerans</td>
</tr>
</tbody>
</table>

T-RF, terminal restriction fragment. Identification is based on the reference list of virtual digests obtained from the in silico MiCA virtual digests using the RDPII database (release 9.51, update 51, bacterial SSU 16S rRNA) in which the used primers and restriction enzyme were inserted.

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DISCUSSION

The aim of the present study was to test whether the nature of the dietary carbohydrate source influences the development of NEC in formula-fed preterm pigs. In our previous studies in preterm pigs, we found that feeding formula induces NEC secondary to mucosal atrophy and reduced digestive function, whereas feeding colostrum largely prevented NEC (5, 41). An important difference in the nutrient composition between the diets used in previous studies was that the main carbohydrate source in colostrum is lactose, whereas our infant formula contained mainly maltodextrin. The ability of the preterm piglet to digest and tolerate maltodextrin in vivo was unknown. The pig has a relatively low activity of enzymes that digest maltodextrin compared with lactase and, thus, might be expected to have digestive problems when fed maltodextrin diets. However, we previously showed that full-term pigs have few major digestive problems and do not develop NEC when fed the same maltodextrin-based formula (2, 32, 41, 42, 51). This led us to hypothesize that carbohydrate maldigestion could trigger bacterial overgrowth and fermentation and serve as an initial event inducing a mucosal proinflammatory response. Testing this hypothesis seemed important, given the studies by Butel and others (10, 22–24, 50) showing that lactose feeding induced NEC in lactase-deficient birds, coupled with speculation that maldigestion of lactose in preterm infants may predispose to NEC. Our present results indicate that the preterm pig intestine has a very limited capacity to digest maltodextrin, probably due to relatively low maltase and sucrase activities. We also show that maldigestion of maltodextrin corresponded with altered assemblages and metabolic activities of the resident bacteria and increased incidence and severity of NEC compared with pigs fed a lactose-based formula.

The specific activities of the major intestinal hydrolase enzymes in our preterm pigs confirm a much higher activity of lactase than either maltase or sucrase in the prenatal pig. Lactase is the main intestinal enzyme that hydrolyzes ingested lactose to glucose and galactose. The maltodextrin used in these studies represents the hydrolytic product of starch and
mannitol absorption, which is an indicator of passive absorption and absorptive surface area of the intestine, was also lower after 6 h of feeding the maltodextrin-based formula. Collectively, these findings suggest that the maltodextrin-based formula elicited a very rapid and pronounced adverse decline in epithelial absorption. Additionally, paracellular absorption does not contribute significantly to sugar absorption, inasmuch as this would have resulted in similar concentrations of sugars in the portal blood during the in vivo studies. Interestingly, in experiment 2, the higher in vivo absorption of glucose than galactose derived from lactose is similar to previous findings in conscious, preterm pigs after a period of TPN (8) and corresponds with the lower affinities of the sodium-glucose-linked transporter-1 and GLUT2 for galactose than for glucose (13, 38).

Because of the short exposure period, it is unlikely that the lower in vivo absorption of galactose after just 6 h of feeding in the maltodextrin-based formula. Collectively, these findings suggest that the maltodextrin-based formula elicited a very rapid and pronounced adverse decline in epithelial absorption. Additionally, paracellular absorption does not contribute significantly to sugar absorption, inasmuch as this would have resulted in similar concentrations of sugars in the portal blood during the in vivo studies. Interestingly, in experiment 2, the higher in vivo absorption of glucose than galactose derived from lactose is similar to previous findings in conscious, preterm pigs after a period of TPN (8) and corresponds with the lower affinities of the sodium-glucose-linked transporter-1 and GLUT2 for galactose than for glucose (13, 38).

Because of the short exposure period, it is unlikely that the lower in vivo absorption of galactose after just 6 h of feeding in the maltodextrin group than the lactose group is caused by lower carrier-mediated absorption. The lower in vivo absorption of glucose is more likely caused by activities of sucrase and maltase in the maltodextrin group that are lower than lactase activity in the lactose group and, thereby, release of less monosaccharide for absorption. The consequence of the combined lower hydrolysis of maltodextrin and absorption of glucose is higher luminal concentrations of fermentable substrate in the maltodextrin group.

Our measurements of in vivo galactose absorption are based on incremental increases in plasma galactose and provide indirect estimates of intestinal sodium-glucose-linked transporter-1 transport capacities. The lack of differences in in vivo galactose absorption after 36 h of enteral feeding between the maltodextrin and lactose groups coincides with similar rates of in vitro tissue-specific glucose uptake in the proximal small intestine. Although intact tissue uptakes were lower in the middle and distal regions of the small intestine of the pigs fed the maltodextrin-based formula, the majority of sugar absorption occurs in the proximal region. Importantly, despite a lack of overt signs of NEC, in vivo and ex vivo aldohexose absorption by the pigs infused with the lactose-based formula after 36 h was <20% of that measured for colostrum-fed pigs in our previous study (6).

Bacteria colonize the GIT immediately after delivery, and high densities of a relatively simple assemblage of bacteria were already established at the start of enteral feeding in our study. The different assemblages of bacteria detected in the intestine of the pigs fed the maltodextrin- and lactose-based formulas reveal dietary carbohydrate influences on the composition of the resident bacteria. Of specific interest was the T-RF at 611 bp, which likely represents *Weissella* spp. This bacterium is a member of the lactic acid-producing bacteria in the GIT. It accounted for ~22% of the total microbiota in pigs fed the lactose-based formula, but only ~5% in pigs fed the maltodextrin-based formula. The plate count data revealed high densities of lactic acid bacteria in the small intestine and colon, with higher densities in the pigs fed the lactose-based formula. We found that the sequence representing *Leuconostoc/Pseudomonas* spp. and *Streptococcus* spp. was present in greater proportions in pigs fed the maltodextrin-based formula.
Correspondingly, the impact of maltodextrin on brush-border densities are higher and NEC is more likely to develop. Regions of the small intestine and in the colon, where bacterial activity is expected to be more pronounced in the middle and distal regions, leading to extensive gas accumulation, intestinal and abdominal distension, and tissue (pneumatosis intestinalis). The resulting gut distension compromises vascular perfusion of the tissue, leading to ischemia and hypoxia, which contribute to the pathological events leading to NEC. Consistent with this, multiple gas bubbles were often observed in the small intestines and colons of preterm pigs with NEC in this study.

The present study showed that feeding preterm pigs a formula with poorly digested carbohydrates (e.g., maltodextrin) causes a number of GIT changes. These include alterations in the assemblages and metabolic activities of the resident bacteria, leading to extensive gas accumulation, intestinal and abdominal distension, and gut dysfunction and, ultimately, gut inflammation and an increased incidence and severity of NEC. The responses of the bacteria to nondigestible oligosaccharides are expected to be more pronounced in the middle and distal regions of the small intestine and in the colon, where bacterial densities are higher and NEC is more likely to develop. Correspondingly, the impact of maltodextrin on brush-border membrane enzyme activities and glucose uptake was evident in the middle and distal, but not proximal, regions of the small intestine. Importantly, the compromised digestive functions, particularly nutrient uptake, would reduce absorption of water, contributing to the onset of diarrhea.

Perspectives

The aim of the present study was to test the hypothesis that malabsorption of carbohydrates is sufficient to trigger the onset of NEC in preterm pigs, which represents a clinically relevant model of intestinal prematurity. We show that the incidence of NEC was significantly higher in preterm pigs fed a maltodextrin-based formula than in those fed a lactose-based formula. Moreover, we show that the developmental low capacity of intestinal maltase and sucrase markedly limits the digestion of maltodextrin, whereas the relatively high lactase activity enables substantial hydrolysis and absorption of lactose. We postulate that the malabsorption of maltodextrin leads to rapid changes in gut microbiota and fermentation that ultimately trigger an inappropriate intestinal proinflammatory response, resulting in NEC. In contrast to the developmental pattern of carbohydrate enzyme activity in preterm pigs, in human preterm and full-term infants, activities of malabsorption and sucrase are two- and sixfold higher than that of lactase. At 34 wk of pregnancy, specific activities of maltase and sucrase are ~70% of adult human values, whereas specific activity of lactase is only ~30% of that measured at full term at this stage of gestational development. The enzyme profiles for human infants are consistent with evidence of incomplete digestion of lactose in infant formula and sufficient capability of the premature human infant to digest glucose polymers. Corresponding with low lactase activity, preterm human neonates fed 100% lactose have higher breath H2 excretion than those fed a formula with a 50:50 mixture of lactose and glucose polymers. It has been suggested that the incomplete digestion and colonic fermentation of lactose in preterm infants represents a risk factor for NEC, yet this has not been tested in a clinical trial. Therefore, despite the different developmental digestive enzyme profiles between preterm pigs and human infants, we suggest that the present study highlights the potential risk associated with malabsorption of dietary carbohydrate, whether it is lactose or maltodextrin, as an early luminal event in the pathogenesis of NEC. Although we mainly attribute the higher NEC incidence to malabsorption and a general bacterial overgrowth, we cannot exclude the possibility that maltodextrin per se has detrimental effects on intestinal function mediated by changes in intestinal aldohexose transport, the products of specific bacteria, or the products of bacteria that trigger intestinal inflammation. This may be relevant, since considerable evidence shows that human milk is protective against NEC and contains mainly lactose, whereas many formulas fed to preterm infants contain substantial amounts of maltodextrin along with lactose.

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GRANTS

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