Protein-Energy Malnutrition Delays Small-Intestinal Recovery in Neonatal Pigs Infected with Rotavirus[1,2]

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ABSTRACT Infectious diarrheal diseases and protein-energy malnutrition (PEM) are major causes of child morbidity and mortality worldwide. In the present study, PEM was superimposed on rotavirus infection in neonatal pigs to simulate chronic small intestinal stress in malnourished infants with viral gastroenteritis. Two-day-old cesarean-derived pigs ($n = 39$) were allotted to three treatment groups: *1*) noninfected, full-fed; *2*) infected, full-fed; and *3*) infected, malnourished. Two days postinfection, severe diarrhea and weight loss (11%) were accompanied by reductions in villus height (60%) and lactase activity (78%) and increased crypt depth (32%) in infected full-fed compared with noninfected pigs ($P < 0.05$). Malnutrition blunted ($P < 0.05$) increases in crypt depth elicited by rotavirus. By 9 d postinfection, body weight was 59% less, villus height and lactase activity remained lower (50%), and crypt depth remained greater (62%) in infected full-fed compared with noninfected pigs ($P < 0.05$). However, diarrhea began to clear in infected full-fed, but not in infected malnourished pigs. Plasma insulin-like growth factor-I (IGF-I) was reduced 68% and crypt depth was reduced 19% in infected-malnourished compared with infected full-fed pigs ($P < 0.05$). Sixteen days postinfection, full-fed pigs had recovered from rotaviral infection; however, in infected-malnourished pigs, diarrhea and growth stasis persisted, and plasma IGF-I, villus height and alkaline phosphatase activity remained reduced compared with infected full-fed pigs ($P < 0.05$). Overall, PEM prolonged diarrhea and delayed small-intestinal recovery, indicating that nutritional status during diarrhea is essential for recovery from rotaviral enteritis. J. Nutr. 127: 1118–1127, 1997.

KEY WORDS: • *rotavirus* • *malnutrition* • *pigs* • *neonate* • *small intestine*

Diarrheal diseases and protein-energy malnutrition (PEM)[6] are primary causes of child morbidity and mortality worldwide. The World Health Organization estimates that one billion diarrheal episodes occur in infants annually, resulting in 3.3 million deaths (Bern et al. 1992). Of the episodes, 20–35 million occur in the U.S., resulting in more than 200,000 hospitalizations and 300–400 deaths (Centers for Disease Control and Prevention 1992). Rotaviruses are the major cause of infectious diarrhea (Bartlett et al. 1987), accounting for 20% of diarrhea-associated deaths in developing countries and for one third of the hospitalizations for diarrheal illnesses in the U.S. (Lieberman 1994). Rotavirus infections are characterized by viral replication in small intestinal enterocytes (Estes 1990), with subsequent cell lysis and attendant villus blunting (Theil et al. 1978), depressed levels of mucosal disaccharidases (Bishop et al. 1973), watery diarrhea (Theil et al. 1978) and dehydration. Reduced enzymatic and absorptive capacity in the small intestine is thought to result in a malabsorptive-type diarrhea (Argenzio et al. 1990).

Nutritional status can influence diarrhea through at least two mechanisms. First, PEM can reduce the integrity of the intestinal epithelium, facilitating bacterial translocation (Cunningham-Rundles 1994) with subsequent enteritis and diarrhea. Second, epidemiologic evidence in human infants suggests that duration of diarrhea may be prolonged by PEM (Chandra 1983). Chronic PEM impairs epithelial proliferation in crypts in the small intestine, resulting in delayed cellular migration along the crypt-villus axis (Guiraldes and Hamilton 1981). Therefore, structural epithelial repair and restoration of enzymatic and absorptive capacity may be delayed in undernourished infants with enteritis (Butzner et al. 1985).

We postulate that PEM will delay recovery from rotavirus infection and have compared metabolic and intestinal conse-

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[6] Abbreviations used: IGF-I and -II, insulin-like growth factor-I and -II; IGFBP, insulin-like growth factor binding protein; MEM, minimal essential medium; PEM, protein-energy malnutrition.

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quences of rotaviral infection in full-fed and malnourished neonatal piglets in the present study. Small intestinal morphology and digestive enzyme activities were measured to monitor intestinal structure and function. Plasma insulin, glucagon, insulin-like growth factors (IGF)-I and -II, and IGF-binding proteins (IGFBP) were measured as indices of nutritional status (Thissen et al. 1994). Together, the present results improve our understanding of how PEM affects metabolic and cellular mechanisms underlying intestinal recovery from rotavirus. This information should aid in the design of nutritional strategies for the management of infectious diarrhea in malnourished and well-nourished children.

MATERIALS AND METHODS

Animals and diet. The study was approved by the University of Illinois Laboratory Animal Care Advisory Committee and was conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals (NRC 1985). Chemical reagents were purchased from Sigma Chemical (St. Louis, MO) unless specified otherwise. Crossbred pigs [n = 39; Yorkshire × Hampshire; average birth weight 1.21 kg ± 0.24 (SD)] were obtained from four cesarean sections (two cesarean sections in each of two periods). Pigs were transferred into a containment area that consisted of separated suites of four cubicles each with independent airflows, i.e., positive air pressure in one suite for noninfected pigs and negative air pressure in one suite for infected pigs. Diets were mixed in a third suite. Pigs were housed in metabolic cages as described previously (Zijlstra et al. 1996). Cubicles were disinfected prior to use with consecutive washes of bleach, a virucide/bactericide solution (Nolvasan, Fort Dodge Laboratories, Fort Dodge, IA) and a bactericide solution (Ster-Bac Blu, Ecolab, St. Paul, MN). Metabolic cages and materials used for feeding were sterilized by autoclaving at 120°C for 20 min prior to use.

Pigs were deprived of colostrum and fed formula via a gravity feeding system, similar to the design in McClead et al. (1990). A simulated sow milk diet was prepared by the Mead Johnson Nutritional Group (Evansville, IN) as a nonsterile dry powder. The diet composition (g/kg diet) was as follows: protein, 300; fat, 360; carbohydrate, 251; water, 40; vitamin and mineral premix,[7] 49. The diet contained 22.8 MJ metabolizable energy/kg diet (25.2 MJ gross energy/kg diet). Bovine whey protein was used as protein source, coconut oil and corn oil were used as fat sources (55:45), and lactose was used as carbohydrate source (McClead et al. 1990). The diet met nutritional requirements for growing pigs from birth through 3 wks of age (McClead et al. 1990). The dry diet was reconstituted daily by addition of deionized water (183 g/L) using a blender (Waring Products, New Hartford, CT). Fresh liquid formula was provided at 12-h intervals. The formula was not sterile, but tested negative for potential pathogens.

Virus purification and quantification. Stock rotavirus (Gelberg 1992) was passed through 1-d-old gnotobiotic pigs to amplify the amount of virus and to retain pathogenicity. Feces from infected pigs were collected and centrifuged for 15 min (5000 × g, 4°C). Supernatants were qualitatively assayed for presence of group A specific rotavirus antigen by micro-ELISA (Gelberg et al. 1991), and rotavirus-positive supernatants were centrifuged for 15 min (9000 × g, 4°C). Resultant supernatants were extracted twice with equal volumes of trichlorotrifluoroethane in a Dounce tissue grinder (Wheaton Scientific, Millville, NJ) and centrifuged for 10 min (1750 × g). The aqueous phase of the solvent was extracted twice with equal volumes of minimal essential medium (MEM), and aqueous phases were pooled and centrifuged for 30 min (9000 × g). Virus particles were then pelleted at 20,000 × g. The pellet was homogenized in MEM and refrigerated overnight. The viral suspension was filtered consecutively through 0.45- and 0.22-mm filters for sterilization. Presence of group-A specific rotavirus antigen was confirmed by micro-ELISA, and purified virus was visualized with an electron microscope (Basgall et al. 1988).

Virus titer of stock solution was 2.67×10^{10} foci/L as determined by a focus-forming assay (Wong et al. 1981) with modifications (Rolsma 1995) executed as follows. Monolayers of MA-104 cells in 24-well culture plates were rinsed twice with PBS. One milliliter of serum free medium was added to each well and the plates were incubated at 37°C and 5% CO_2 for 3 h. Virus was treated with 10 mg/L crystallized trypsin for 30 min at 37°C. Serial dilutions of virus suspension were added in triplicate and then the plates were rotated every 15 min for 1 h at 37°C. After washing, plates were incubated with MEM at 37°C and 5% CO_2 for 16–18 h. Plates were then rinsed and fixed with methanol/glacial acetic acid (9:1) for 2 min, and rehydrated subsequently for 5 min in 70% ethanol, 50% ethanol and wash buffer. Virus-containing cells were enumerated by immunocytochemistry using rabbit anti-human rotavirus antibody (Dako, Carpinteria, CA), biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and TrueBlue chromagen (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Experimental protocol. After recovery from cesarean delivery (4–6 h), pigs were trained to nurse from the feeding system. Pigs were drinking independently within 12 h and had free access to formula for 2 d (300–350 mL · kg body $wt^{-0.75} \cdot d^{-1}$). At 1 d of age, pigs were injected subcutaneously with 1 mL of a 100 g/L iron dextran solution (Butler, Columbus, OH). At 2 d of age, pigs were randomly allotted, within litter, to three treatment groups. Group 1 included *noninfected* pigs given undiluted formula, n = 12; Group 2 included infected pigs given undiluted formula, n = 14; Group 3 included infected pigs given 50% diluted formula [diluted with electrolyte solution; 1.91 g KCl, 0.15 g NaCl and 1.65 g Na citrate (tri-sodium)/L of deionized water], n = 13. Following randomization, pigs in groups 2 and 3 were infected with 2 mL of porcine rotavirus inoculum (1 mL stock solution + 1 mL PBS). To prevent differences in nutrient intake between noninfected and infected pigs, formula intake (mL · kg body $wt^{-0.75} \cdot d^{-1}$) for each of the three groups was restricted throughout the remainder of the study, according to the following schedule: 1 d postinfection, 300; 2 d postinfection, 240; 2–12 d postinfection, 240–480 (gradual increase); 12–16 d postinfection, 480. The feeding protocol was based upon feed intake data from rotavirus-infected pigs in previous work (Zijlstra et al. 1994). Pigs were weighed daily and formula consumption was measured twice daily for each pig. Diarrhea was scored daily based on consistency of feces (0, no diarrhea; 1, stiff flowing feces; 2, easy flowing feces; 3, severe, watery diarrhea). On d 2, 9 and 16 postinfection, four pigs per treatment were killed by electrocution followed by exsanguination. Blood was collected in heparinized tubes and the gastrointestinal tract was removed. Blood samples were placed on ice, centrifuged at 3000 × g, and plasma was frozen at −80°C until analyzed.

Plasma analyses. *Insulin and glucagon.* Concentrations of the pancreatic hormones were determined by RIA as described previously (Zijlstra et al. 1996). Intra-assay CV were 3% for insulin and 4% for glucagon.

Insulin-like growth factors. To dissociate IGF-I and -II from their binding proteins, plasma samples were chromatographed, and IGF-I and -II were measured in separate RIA as described (Donovan et al. 1994). The following dilution factors were used: 1:10 to 1:50 for IGF-I analysis and 1:50 for IGF-II analysis. Analyses for each peptide were run within a single assay and intra-assay CV were 4% for IGF-I and 6% for IGF-II.

IGF binding proteins. Plasma samples (3 mL) were separated on SDS-PAGE gels, and IGFBP were analyzed by radioligand blotting, autoradiography and densitometry as described (Donovan et al. 1994).

Intestinal sampling. The small intestine was dissected free of mesenteric attachments and placed on a smooth surface in six parts of equal length. This arrangement allowed collection of tissue at seven equidistant points along the length of the small intestine from the duodenum (segment 1) and proximal jejunum (segment 2) to distal jejunum (segment 6) and distal ileum (segment 7) (Cranwell

[7] Vitamins and mineral mixes adjusted the diet composition (per kg diet) to the following: vitamin A, 3 mg; vitamin D, 0.01 mg; vitamin E, 164 mg; vitamin C, 382 mg; folic acid, 2.1 mg; thiamine, 6 mg; riboflavin, 90 mg; niacin, 82 mg; vitamin B-6, 9.8 mg; vitamin B-12, 0.04 mg; biotin, 1.4 mg; pantothenic acid, 44 mg; vitamin K, 3.3 mg; choline, 5.5 g; calcium, 14 g; phosphorus, 10 g; iodine, 0.25 mg; iron, 11 mg; magnesium, 1.1 g; copper, 22 mg; zinc, 175 mg; manganese, 4.9 mg; selenium, 0.27 mg; chloride, 5.5 g; potassium, 5.5 g; sodium, 2.7 g.

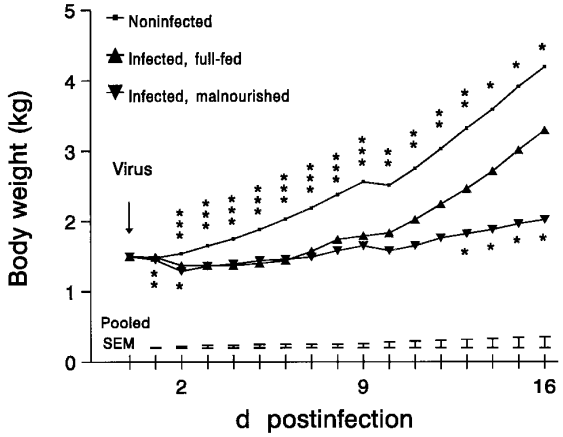

FIGURE 1 Growth curves of noninfected, infected full-fed and infected-malnourished pigs. Infected pigs received a rotavirus inoculum at 2 d of age. Values are least-square means, calculated using body weight at d 2 (1.49 kg) as a covariate. Pooled SEM per day is symbolized as a single error bar; *, differs from infected full-fed, $P < 0.05$; **, differs from infected full-fed, $P < 0.01$; ***, differs from infected full-fed, $P < 0.001$.

and Moughan 1989). Thus, regardless of animal size, tissues were collected from the same relative site along the small intestine. Intestinal content was removed from tissue samples with mild saline washes. Tissue samples (~ 3 cm) from each segment were weighed and either frozen in liquid nitrogen for IGFBP analysis or immersed in 10% neutral buffered formalin for histological analysis. Additional tissue samples (7–15 cm), immediately distal to each segment division, were measured for length and weighed. Mucosa was scraped from these samples with a clean microscope slide, weighed and used for subsequent determination of mucosal enzyme activities. Intestinal samples for IGFBP and enzyme analyses were stored at −70°C until assayed.

Small intestine analyses. *Morphometry.* Tissue samples were processed for light microscopy, and nine villi and nine crypts were measured for each intestinal segment as described previously (Zijlstra et al. 1994). Subsequently, means for villus length and crypt depth were calculated per segment.

Mucosal enzyme activities. Mucosal samples were processed and analyzed for disaccharidases [lactase (EC 3.2.1.23), maltase (EC 3.2.1.20), leucine aminopeptidase (EC 3.4.11.1), alkaline phosphatase (EC 3.1.3.1)], and protein as described (Zijlstra et al. 1994). Results were expressed as specific activities (units per gram or milligram protein). For disaccharidases, 1 unit was defined as the activity that liberates 1 μmol glucose per minute per gram of protein. For other brush border enzymes, 1 unit was defined as the activity that hydrolyzes 1 μmol substrate per minute per gram (leucine aminopeptidase) or milligram (alkaline phosphatase) of protein.

IGF binding proteins. Whole-tissue samples (mucosa + underlying muscle layers) were homogenized (100 g/L) in Laemmli sample buffer (100 g/L glycerol, 20 g/L SDS, 62.5 mmol/L Tris-HCl, pH 6.8) containing 8.3 g/L Triton X-100, 0.003 mmol/L phenylmethylsulfonylfluoride, 0.041 g/L iodoacetic acid, 0.17 g/L trypsin inhibitor and 0.17 g/L EDTA. Protein content was determined in the homogenate by the method of Lowry et al. (1951) with modifications (Hartree 1972) using bovine serum albumin as a standard. Homogenate samples (250 mg protein) were separated on SDS-PAGE gels, and IGFBP were analyzed by radioligand blotting as described (Donovan et al. 1994).

Statistical analyses. Data were analyzed according to a randomized complete-block design (blocked by litter) using the General Linear Models procedure of the SAS statistical package (SAS 1985). Infected pigs fed undiluted formula (group 2; infected full-fed: d 2, $n = 5$; d 9, $n = 5$; d 16, $n = 4$) were compared by one-way ANOVA with either noninfected pigs (group 1: d 2, $n = 4$; d 9, $n = 4$; d 16, $n = 4$) for a rotavirus infection effect or with infected-malnourished pigs (group 3: d 2, $n = 4$; d 9, $n = 5$; d 16, $n = 4$) for a nutrition effect within infected pigs (Steel and Torrie 1980). For analysis of body weight data, body weight at d 2 was used as a covariate. Significance of differences was calculated using the LSMEANS statement, and results are presented as least-square means ± pooled SEM. Differences were considered significant when $P < 0.05$. Instances in which $P < 0.1$ are discussed as trends.

RESULTS

Animal observations. Body weight of 2-d-old pigs was 1.5 kg ± 0.3 (SD) at the time of infection, with no difference among treatment groups. No clinical signs of rotavirus infection were observed in noninfected pigs; personnel routing and sanitization procedures prevented noninfected pigs from contracting rotavirus. Rotavirus infection resulted in weight loss in both full-fed and malnourished infected pigs by 2 d postinfection (**Fig. 1**). Noninfected pigs gained more weight over the whole study period than infected full-fed pigs; however, after 11 d postinfection, the rate of gain did not differ between the two groups. Infected pigs began to gain weight 5 d postinfection. Infected-malnourished pigs gained less weight per day than infected full-fed pigs at 13 d postinfection and throughout the remainder of the study ($P < 0.05$). Feed intake was restricted after inoculation with rotavirus at 2 d of age to prevent major differences in protein and energy intake between noninfected and infected full-fed pigs (**Fig. 2**). Nutrient intake was reduced 50% for infected-malnourished pigs; however, daily intakes for water, sodium, potassium and chloride were similar to infected full-fed pigs, because formula was diluted 50% with an electrolyte solution. Diarrhea was not observed in any treatment group prior to inoculation with rotavirus (**Fig. 3**). Rotavirus infection, however, resulted in severe, watery diarrhea within 2 d, which started to subside at d 9 in full-fed pigs. In malnourished pigs infected with rotavirus, diarrhea persisted throughout the experiment (Fig. 3).

Plasma insulin and glucagon radioimmunoassay. Rotavirus infection alone did not affect plasma insulin or glucagon concentrations (**Table 1**) or the insulin:glucagon ratio (not shown); however, malnutrition did. Plasma glucagon concen-

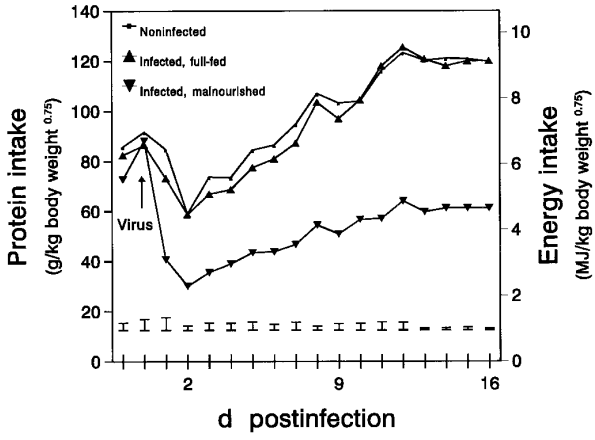

FIGURE 2 Daily protein and (metabolizable) energy intake for noninfected, infected full-fed and infected-malnourished pigs. Values are least-square means, and pooled SEM per day is symbolized as a single error bar. Intakes were calculated using the following assumptions: *1*) diet was reconstituted as follows: 183 g diet + 1 L water = 1.2 L formula; *2*) diet contained 300 g protein and 22.82 MJ metabolizable energy per kg diet; and *3*) formula intake was restricted as follows (mL · kg body weight$^{-0.75}$ · d^{-1}): 300, 1 d postinfection; 240, 2 d postinfection; 240–480 (gradual increase), 2–12 d postinfection; 480, 12–16 d postinfection. Although nutrient intake was reduced for infected-malnourished pigs, daily water intake was similar to infected full-fed pigs, because formula was diluted 50% with an electrolyte solution.

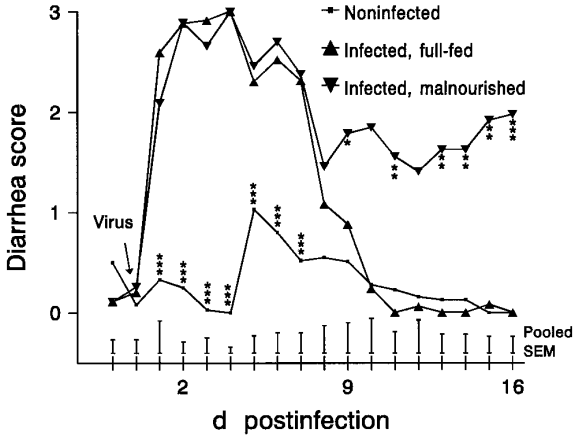

FIGURE 3 Daily diarrhea score for noninfected, infected full-fed and infected-malnourished pigs (0, no diarrhea; 1, stiff flowing feces, 2, easy flowing feces; 3, severe, watery diarrhea). Values are least-square means, and pooled SEM per day is symbolized as a single error bar; *, differs from infected full-fed, $P < 0.05$; **, differs from infected full-fed, $P < 0.01$; ***, differs from infected full-fed, $P < 0.001$.

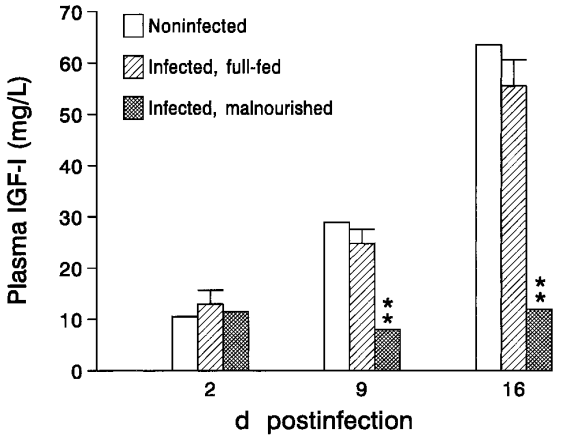

FIGURE 4 Plasma insulin-like growth factor-I (IGF-I) concentrations at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs. Values are least-square means, and pooled SEM per time point is symbolized as a single error bar; *, differs from infected full-fed, $P < 0.05$; **, differs from infected full-fed, $P < 0.01$.

trations of infected-malnourished pigs were twofold higher at 2 d postinfection ($P < 0.1$) and were 45% less at 9 d postinfection than those of infected full-fed pigs ($P < 0.1$). At 16 d postinfection, plasma insulin concentrations of infected-malnourished pigs were 83% less than those of infected full-fed pigs and the plasma insulin:glucagon ratio (not shown) was 86% less ($P < 0.05$).

Plasma insulin-like growth factors and binding proteins. Rotavirus infection alone did not affect plasma IGF-I concentrations ($P > 0.1$; **Fig. 4**), however, malnutrition did. Plasma IGF-I concentrations of infected-malnourished pigs were 68% less at 9 d postinfection and 78% less at 16 d postinfection than those of infected full-fed pigs ($P < 0.01$). Plasma IGF-I concentrations in noninfected pigs increased fivefold during the 2 wk of the study.

Rotavirus infection alone did not affect plasma IGF-II concentrations ($P > 0.1$; Table 1). Plasma IGF-II concentrations of infected-malnourished pigs were 31% less than those of infected full-fed pigs at 9 d postinfection ($P < 0.06$; Table 1). Plasma IGFBP-3 and IGF-I followed similar patterns (**Table 2**), i.e., no effect of rotavirus infection, and plasma IGFBP-3 concentrations of infected-malnourished pigs were 86% less at 9 d postinfection ($P < 0.05$; **Fig. 5**A) and 81% less at 16 d postinfection ($P < 0.001$) than those of infected full-fed pigs. Plasma IGFBP-2 concentrations of infected full-fed pigs were 40% less than in noninfected pigs at 16 d postinfection ($P < 0.001$; Table 2). Plasma IGFBP-2 concentrations of infected-malnourished pigs were 26% higher than those of infected full-fed pigs at 16 d postinfection ($P < 0.05$). Plasma IGFBP-1 and -4 concentrations were similar among treatment groups.

TABLE 1

Plasma insulin, glucagon and insulin-like growth factor-II (IGF-II) concentrations at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs[1,2]

	Insulin	Glucagon	IGF-II
	pmol/L	*ng/L*	*mg/L*
2 d Postinfection			
Noninfected	13.2	136.0	111.1
Infected/full-fed	18.0	120.0	163.2
Infected/malnourished	31.6	354.8	190.1
Pooled SEM	6.1	73.1	26.2
9 d Postinfection			
Noninfected	25.4	76.4	148.1
Infected/full-fed	17.9	121.5	128.5
Infected/malnourished	3.3	67.1	88.4
Pooled SEM	7.2	19.6	14.9
16 d Postinfection			
Noninfected	19.9	43.0	163.8
Infected/full-fed	38.2	43.9	147.4
Infected/malnourished	6.4*	93.3	117.7
Pooled SEM	5.9	28.5	24.7

1 Values are least-square means.
2 Blood was collected 13–16 h after the last provision of fresh formula.
* Differs from infected full-fed, $P < 0.05$.

Intestinal morphometry. Relative to noninfected pigs, rotavirus infection reduced total small intestine weight 24% by 2 d postinfection ($P < 0.01$; **Fig. 6**), resulting predominantly from a 53% reduction of mucosal weight (weight/cm intestine) in the medial small intestine ($P < 0.05$; Fig. 6). At 9 d postinfection, total small intestine weight did not differ in noninfected and infected full-fed pigs; however, mucosal weight was reduced 73% in infected pigs in the medial small intestine ($P < 0.05$; Fig. 6). Total small intestine weight of infected-malnourished pigs was 25% less than that of infected full-fed pigs by 9 d postinfection ($P < 0.01$; Fig. 6), resulting predominantly from a 15% reduction of muscle (total weight − mucosa weight) specific weight in the proximal and medial small intestine ($P < 0.05$; Fig. 6). At 16 d postinfection, total small intestine relative weight did not differ in noninfected and infected full-fed pigs. Total small intestine relative weight of infected-malnourished pigs was 25% less than that of infected full-fed pigs by 9 d postinfection and 38% less by 16 d postinfection ($P < 0.01$; Fig. 6), resulting predominantly from a 45% reduction of mucosal specific weight and a 26% reduction of muscle specific weight in the proximal small intestine ($P < 0.05$; Fig. 6).

Rotavirus infection decreased villus height 58% throughout

TABLE 2

Insulin-like growth factor binding proteins (IGFBP) in plasma and small intestine tissue at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs[1]

	Plasma IGFBP				Small intestine tissue IGFBP			
	1	2	3	4	1	2	3	4
	Arbitrary densitometric units				*Arbitrary densitometric units*			
2 d Postinfection								
Noninfected	196	331	470	128	25**	47	ND	28*
Infected/full-fed	224	289	419	104	58	101	ND	69
Infected/malnourished	233	294	444	281	50	125	ND	48
Pooled SEM	46	49	112	75	6	25	—	8
9 d Postinfection								
Noninfected	130	381	650	180	24	40	ND	32
Infected/full-fed	209	406	495	90	36	50	ND	40
Infected/malnourished	234	478	66*	65	49	69	ND	37
Pooled SEM	75	28	73	67	6	7	—	4
16 d Postinfection								
Noninfected	20	671***	973	59	ND	ND	ND	ND
Infected/full-fed	32	402	927	25	7	18	ND	ND
Infected/malnourished	214	508*	178***	16	7	24	ND	ND
Pooled SEM	48	18	45	11	2	4	—	—

[1] Values are least-square means. Means are compared within plasma and small intestine tissue. The two IGFBP-3 bands were combined for density measurements.

ND = not detectable. * Differs from infected full-fed, $P < 0.05$. ** Differs from infected full-fed, $P < 0.01$. *** Differs from infected full-fed, $P < 0.001$.

the jejunum and ileum by 2 d postinfection ($P < 0.05$; **Fig. 7***A*). Nine days postinfection, villi remained shorter in infected full-fed pigs compared with noninfected pigs, i.e., 34% shorter in the proximal half ($P < 0.05$) and 63% shorter in the distal half ($P < 0.001$) of the small intestine (Fig. 7*B*). Heights of jejunal and ileal villi were similar by d 16 postinfection in infected full-fed and noninfected pigs (Fig. 7*C*). Villus height of infected-malnourished pigs was 42% less than that of infected full-fed pigs in the distal small intestine at 16 d postinfection ($P < 0.05$).

Rotavirus infection increased crypt depth 32% in the distal small intestine by 2 d postinfection, and 62% in the medial and distal small intestine at 9 d postinfection ($P < 0.01$; Fig. 7*A*). Crypt depth in the distal small intestine of infected-malnourished pigs was 18% less than that of infected full-fed pigs at 2 d postinfection, and 19% less in the medial and distal small intestine at 9 d postinfection ($P < 0.05$). By 16 d postinfection, crypt depth measurements did not differ among treatments (Fig. 7*C*).

Mucosal enzyme activity. Because enzyme activities in segment 1 were similar among treatment groups in a pilot experiment (unpublished data), only segments 2 through 7 were analyzed for enzyme activity in this study. Rotavirus infection reduced lactase specific activity 78% throughout the small intestine by 2 d postinfection, and 61% in the distal small intestine at 9 d postinfection ($P < 0.05$; **Table 3**). At 16 d postinfection, lactase specific activity of infected-malnourished pigs was twice that of infected full-fed pigs in segment 3 of the small intestine and was 62% less in segment 6 ($P < 0.05$). Maltase specific activity did not differ among treatments at 2 and 9 d postinfection (**Table 4**). By 16 d postinfection, maltase specific activity of infected full-fed pigs was 75% less than that of noninfected pigs in the distal small intestine; maltase specific activity of infected-malnourished pigs was 69% higher than that of infected full-fed pigs in segment 3 of the small intestine ($P < 0.05$). Rotavirus infection reduced leucine aminopeptidase specific activity 42% in the medial small intestine and 26% in the distal small intestine by 2 d postinfection, and 52% in the proximal and distal small intestine by 9 d postinfection ($P < 0.05$; **Table 5**). Rotavirus infection reduced alkaline phosphatase specific activity 85% in the medial and distal small intestine by 2 d postinfection, and 72% in the medial small intestine at 9 d postinfection ($P < 0.05$; **Table 6**). By 16 d postinfection, alkaline phosphatase specific activity of infected-malnourished pigs was 76% less than that of infected full-fed pigs in the distal small intestine ($P < 0.05$).

Intestinal insulin-like growth factor binding proteins. As shown in Fig. 5*B*, intestinal and plasma IGFBP migrated at similar molecular weights through SDS-PAGE gels. The identities of IGFBP-2 and -4 were further verified by immunoblotting (not shown). At 2 d postinfection, intestinal IGFBP-1 ($P < 0.01$, Table 2) and IGFBP-4 ($P < 0.05$) concentrations were doubled by rotavirus infection, with no effect of malnutrition in infected pigs. At 9 d postinfection, intestinal IGFBP-2 concentration of infected-malnourished pigs was 38% higher than that of infected full-fed pigs (Fig. 5*B*; $P < 0.1$, Table 2). At 16 d postinfection, intestinal IGFBP-1 and IGFBP-2 concentrations were higher in rotavirus-infected pigs than in noninfected pigs ($P < 0.1$). IGFBP-3 was not detected in any of the intestinal samples, indicating minimal contamination of the intestine with blood.

DISCUSSION

In the present study, PEM was superimposed on rotavirus infection in neonatal pigs to simulate chronic intestinal stress in malnourished infants with viral gastroenteritis. Metabolic and intestinal responses of rotavirus-infected malnourished pigs were compared with those of infected full-fed pigs to determine specific effects of malnutrition on recovery from rotavirus. In addition, responses of infected full-fed pigs were compared with pair-fed noninfected pigs to distinguish specific metabolic and intestinal effects of rotavirus infection. Rotavirus infection caused diarrhea, reduced body weight gain, damaged the structure of the intestine, reduced intestinal enzyme activities and increased intestinal IGFBP, but did not alter

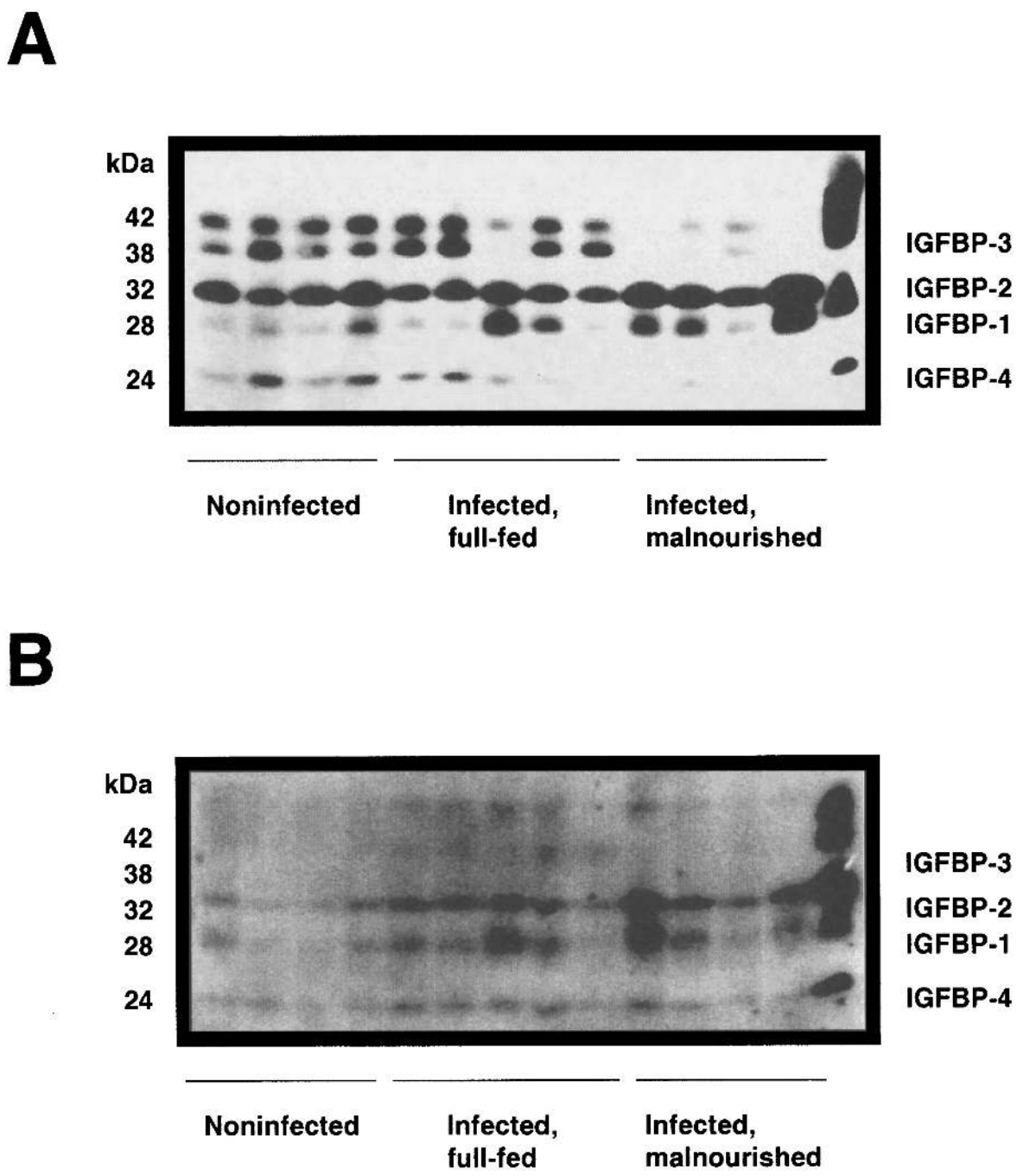

FIGURE 5 Radioligand blots for insulin-like growth factor binding proteins (IGFBP) in plasma (*panel A*) and small intestine tissue (*panel B*) at 9 d postinfection for noninfected, infected full-fed and infected-malnourished pigs. Results of densitometric analyses of both variables for each day postinfection are presented in Table 2. Rat plasma was used as control for the blot in *panel A* and pig plasma for the blot in *panel B* (right lanes).

plasma insulin or IGF-I concentrations. As postulated, recovery from rotaviral enteritis was delayed by PEM, indicated by prolonged diarrhea and delayed recovery of intestinal structure and function. Reduced plasma insulin and IGF-I concentrations verified PEM.

Rotavirus induced severe, watery diarrhea in infected pigs within 2 d, with a subsequent growth stasis for 1 wk. Diarrhea cleared by 10 d postinfection in infected full-fed pigs but not in infected-malnourished pigs, demonstrating that nutrient intake during rotaviral enteritis affects the duration of diarrhea. These data are consistent with recent evidence that early refeeding of children with enteritis decreases the duration of diarrhea (Provisional Committee on Quality Improvement 1996). Nutritional management of infants with infectious diarrhea after initial rehydration remains controversial (Gracey 1996), partly because of the view that malabsorption is a major mechanism underlying rotaviral diarrhea. Impaired digestion and absorption are thought to result from a reduced villus surface area composed predominantly of immature, undifferentiated cells. Reduced enzymatic activities would result in undigested material within the small intestine causing malabsorptive-type diarrhea (Perman 1985). Following the malabsorptive diarrhea paradigm, rotaviral and other diarrheal diseases are commonly treated by "bowel rest," i.e., reduction of luminal nutrients in the gastrointestinal tract during the diarrheal episode (Lieberman 1994). An opposing view is to resume

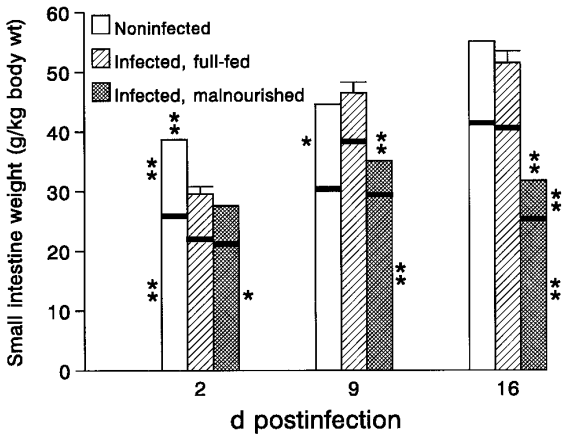

FIGURE 6 Total small intestine relative weight (g/kg body weight) at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs. The dividers within graph bars indicate the fraction of total small intestine weight that is muscle (below divider) or mucosa (above divider). Data are pooled across intestinal segments. Effects of rotavirus or malnutrition on specific intestinal regions are noted in Results. Values are least-square means, and pooled SEM per time point is symbolized as a single error bar. Asterisks above graph bars indicate significant differences in total small intestinal weight between noninfected and infected, full-fed animals, or between infected, full-fed and infected, malnourished animals. Asterisks to the right or left of graph bars and above (mucosa) or below (muscle) the dividing lines indicate significant differences in either of those fractions for the same treatment comparisons; **, differs from infected full-fed, $P < 0.01$.

normal feeding as soon as possible to avoid consequences of reduced nutrient intake (Centers for Disease Control and Prevention 1992) because nutrients are absorbed even during diarrhea (Lieberman 1994). The malabsorptive diarrhea paradigm is partially supported by present evidence that diarrhea in infected pigs at 2 d postinfection coincided with reductions of small intestine villus surface area and specific activities of mucosal enzymes. However, both metabolic and intestinal data from the present study bring into question the extent to which malabsorption contributes to rotaviral diarrhea.

The peptide IGF-I has been identified as a useful systemic indicator of nutritional status (Buonomo and Baile 1991, Thissen et al. 1994). Plasma IGF-I, IGFBP and insulin concentrations were similar between noninfected and infected full-fed pigs, indicating that nutritional status was not compromised in infected pigs, and therefore, that digestion and absorption may not have been impaired by rotaviral diarrhea. In infected full-fed pigs, a reduction in the severity of diarrhea (7–9 d postinfection) coincided with the resumption of weight gain, and complete clearance of diarrhea at 10 d postinfection corresponded with a rate of body weight gain similar to noninfected pigs. Thus, the present data demonstrate that providing nutrients to rotavirus-infected piglets during the diarrheal episode does improve nutritional status and contributes to more rapid recovery of weight gain.

Plasma IGF-I and IGFBP-3 concentrations were reduced in infected-malnourished compared with infected full-fed pigs at 9 and 16 d postinfection, verifying malnutrition. Plasma IGF-II, which is generally less sensitive to compromised nutritional status than IGF-I (Thissen et al. 1994), was also reduced at 9 d postinfection. That response may reflect a more rapid clearance of IGF-II as a result of the large reduction (86%) in its primary carrier protein (IGFBP-3; Thissen et al. 1992) in response to malnutrition. Plasma insulin concentrations indicated malnutrition at 16 d postinfection but not at 9 d postinfection. Insulin is likely to be more sensitive than IGF-I to time elapsed between last meal and blood sampling, because insulin, unlike IGF-I, is not bound to binding proteins. Thus, plasma IGF-I concentration appears to be a more useful indicator of chronic nutritional stress than plasma insulin.

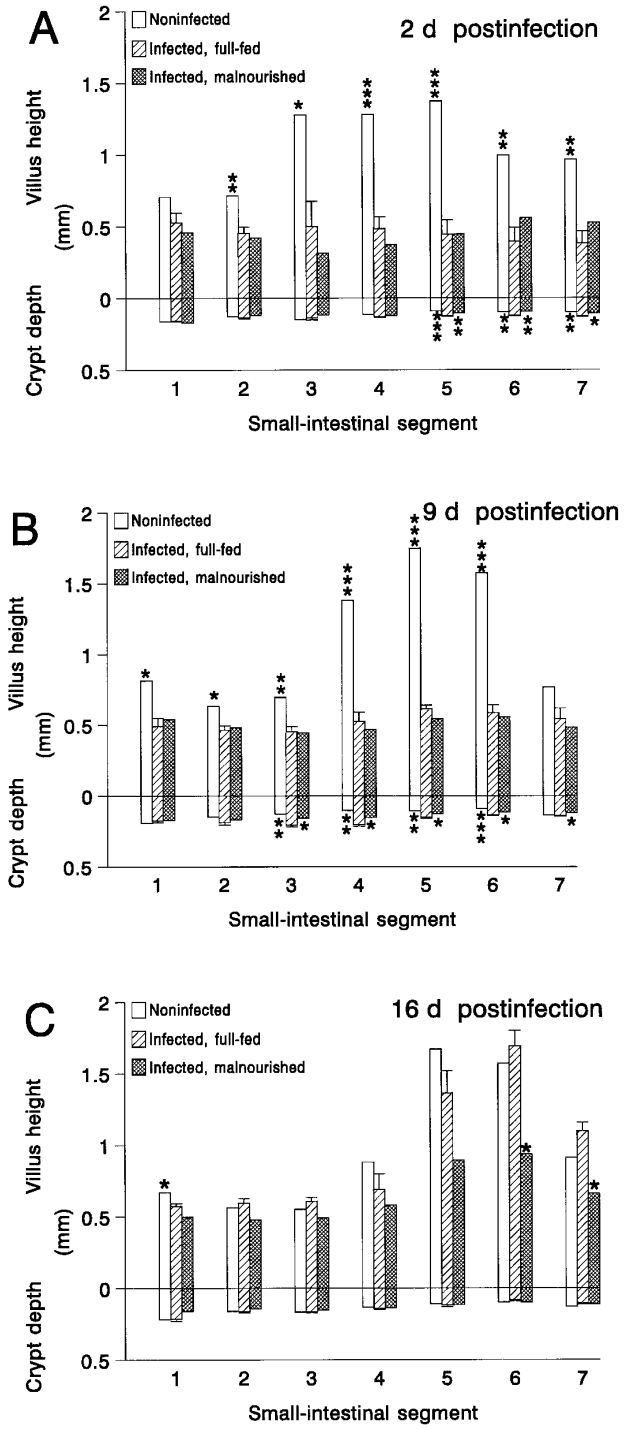

FIGURE 7 Villus height and crypt depth at 2 d (*panel A*), 9 d (*panel B*) and 16 d (*panel C*) postinfection for noninfected, infected full-fed, and infected-malnourished pigs for seven equally spaced segments of the small intestine; segment 1 is proximal, segment 7 is distal. Values are least-square means, and pooled SEM per segment is symbolized as a single error bar; *, differs from infected full-fed, $P < 0.05$; **, differs from infected full-fed, $P < 0.01$; ***, differs from infected full-fed, $P < 0.001$.

TABLE 3

Mucosal lactase specific activity at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs for six of seven equally spaced segments of the small intestine[1,2]

Segment	2	3	4	5	6	7
			$\mu mol \cdot min^{-1} \cdot g\ protein^{-1}$			
2 d Postinfection						
Noninfected	113.2	192.3**	163.4*	145.2**	88.4*	65.8**
Infected/full-fed	66.0	49.5	44.8	29.9	22.8	5.9
Infected/malnourished	178.8	76.2	78.9	60.9	47.5	16.3
Pooled SEM	48.4	26.1	24.7	18.3	19.0	8.2
9 d Postinfection						
Noninfected	75.6	90.8	136.9	134.3	93.3*	53.6*
Infected/full-fed	67.6	81.7	63.4	59.4	37.9	19.9
Infected/malnourished	104.5	77.0	51.6	42.8	21.1	16.0
Pooled SEM	24.3	26.1	36.7	31.8	11.9	6.8
16 d Postinfection						
Noninfected	121.2	98.7	127.3	121.1	99.5	15.2
Infected/full-fed	117.2	72.9	109.0	143.2	126.9	44.9
Infected/malnourished	132.8	152.5**	112.8	118.6	48.4*	60.1
Pooled SEM	7.2	10.0	15.6	17.5	12.6	14.1

[1] Values are least-square means.
[2] Segment 2 is proximal, segment 7 is distal.
* Differs from infected full-fed, $P < 0.05$. ** Differs from infected full-fed, $P < 0.01$.

The intestine is considered one of the major targets of IGF action (Zeeh et al. 1995). Receptors for IGF are located on both apical and basal membranes of enterocytes (Morgan et al. 1996). Therefore, both orally and systemically administered IGF could modulate structure and function of the small intestine. Recent evidence indeed indicates that circulating IGF-I can regulate protein synthesis in intestinal mucosa (Lo et al. 1996). Pigs in the present study did not receive colostrum, and exogenous IGF-I was not added to the formula. Thus, reduced plasma IGF-I in infected-malnourished pigs, compared with infected full-fed pigs, could partially explain delayed small-intestinal recovery following rotavirus infection. Experimental colitis in rats increased IGF-I binding to IGFBP in muscle underlying the intestinal epithelium, suggesting an important role for IGFBP in modulating local IGF effects during intestinal inflammation and repair (Zeeh et al. 1995). In the present study, intestinal IGFBP were increased by rotavirus infection, supporting that possibility. In addition, intestinal IGFBP-2 concentrations were higher in infected-malnourished than infected full-fed pigs, indicating that IGFBP might modulate IGF-I effects within the tissue in response to nutritional status. Additional studies are required to determine the cellular source of intestinal IGFBP. Nevertheless, the present results suggest for the first time a potentially important role for IGF-

TABLE 4

Mucosal maltase specific activity at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs for six of seven equally spaced segments of the small intestine[1,2]

Segment	2	3	4	5	6	7
			$\mu mol \cdot min^{-1} \cdot g\ protein^{-1}$			
2 d Postinfection						
Noninfected	17.4	10.8	5.9	6.1	4.8	6.7
Infected/full-fed	13.4	11.5	12.3	9.2	8.4	7.6
Infected/malnourished	34.8	11.3	6.2	5.1	6.1	8.0
Pooled SEM	8.7	2.9	3.1	2.1	3.1	1.7
9 d Postinfection						
Noninfected	52.9	74.9	7.2	7.0	6.6	10.5
Infected/full-fed	44.7	78.5	28.1	18.9	8.9	11.3
Infected/malnourished	39.7	45.8	54.4	32.7	18.5	14.5
Pooled SEM	10.7	20.0	9.3	6.6	3.7	5.0
16 d Postinfection						
Noninfected	115.2	119.1	47.4	9.3	11.2	16.8*
Infected/full-fed	113.1	94.7	91.9	10.7	13.3	4.3
Infected/malnourished	120.1	160.3*	41.5	5.2	10.7	14.9
Pooled SEM	11.2	9.4	24.2	3.5	2.2	2.4

[1] Values are least-square means.
[2] Segment 2 is proximal, segment 7 is distal.
* Differs from infected full-fed, $P < 0.05$.

TABLE 5

Mucosal leucine aminopeptidase specific activity at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs for six of seven equally spaced segments of the small intestine[1,2]

Segment	2	3	4	5	6	7
			$\mu mol \cdot min^{-1} \cdot g\ protein^{-1}$			
2 d Postinfection						
Noninfected	6.2	13.0**	11.7*	16.5**	19.0*	18.5***
Infected/full-fed	5.9	4.7	5.6	3.6	5.4	5.2
Infected/malnourished	12.7	4.2	6.8	7.5	10.7	6.5
Pooled SEM	3.7	1.4	1.5	2.4	3.4	1.0
9 d Postinfection						
Noninfected	5.6*	6.5	10.8	18.9**	22.3***	17.9
Infected/full-fed	3.3	6.7	9.4	9.5	10.2	11.7
Infected/malnourished	5.6*	6.8	8.9	9.6	11.2	11.9
Pooled SEM	0.6	1.5	1.1	1.9	0.6	2.3
16 d Postinfection						
Noninfected	7.2*	7.2	12.3	17.5	26.1	14.9*
Infected/full-fed	4.3	4.7	7.5	18.5	26.5	25.4
Infected/malnourished	7.1	8.0	7.8	14.1	16.8	14.6*
Pooled SEM	0.5	0.7	1.1	2.1	2.1	1.7

[1] Values are least-square means.
[2] Segment 2 is proximal, segment 7 is distal.
* Differs from infected full-fed, $P < 0.05$. ** Differs from infected full-fed, $P < 0.01$. *** Differs from infected full-fed, $P < 0.001$.

I and its binding proteins in intestinal recovery from viral insults. Evidence that intestinal IGF physiology is modulated in response to malnutrition is particularly intriguing.

Group A rotavirus infects differentiated enterocytes towards the villus tips in the small intestine, leading to cell death and eventual villus atrophy (Theil et al. 1978). Histomorphological analysis verified disruption of the epithelium (data not shown) and villus atrophy in the present study. Immediately following rotavirus infection, stem cells in the crypts undergo hyperplasia resulting in crypt elongation. In the present study, crypt elongation in the small intestine of infected-malnourished pigs was diminished relative to infected full-fed pigs, indicating that PEM reduced stem cell division, thereby limiting the production of fully developed enterocytes. Intestinal recovery was complete by 16 d postinfection in full-fed but not in malnourished pigs, based on villus height data.

Mucosal enzymes can also serve as markers for small intestine damage. Alkaline phosphatase is a villus tip marker (Collins et al. 1988); lactase activity is expressed predominantly in the upper villus (James et al. 1987), and maltase activity is located toward the base of the villus (James et al. 1987). Lactase, leucine aminopeptidase and alkaline phosphatase specific activities, but not maltase specific activity, were reduced by rotavirus infection. These results provide additional verification that rotavirus infects

TABLE 6

Mucosal alkaline phosphatase specific activity at 2, 9 and 16 d postinfection for noninfected, infected full-fed and infected-malnourished pigs for six of seven equally spaced segments of the small intestine[1,2]

Segment	2	3	4	5	6	7
			$\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$			
2 d Postinfection						
Noninfected	0.2	2.2	1.4*	2.3*	1.5**	1.1**
Infected/full-fed	0.4	0.4	0.3	0.2	0.2	0.2
Infected/malnourished	1.5	0.3	0.6	0.5	0.4	0.3
Pooled SEM	0.6	0.9	0.3	0.5	0.3	0.2
9 d Postinfection						
Noninfected	0.2	0.2	1.2	2.7*	1.6	1.0
Infected/full-fed	0.2	0.6	0.7	0.8	0.9	0.7
Infected/malnourished	0.3	0.5	0.5	0.9	0.7	0.8
Pooled SEM	0.1	0.2	0.2	0.5	0.2	0.2
16 d Postinfection						
Noninfected	0.3	0.4	0.8	3.1	2.5	0.9
Infected/full-fed	0.4	0.3	0.4	3.8	3.6	1.5
Infected/malnourished	0.3	0.3	0.6	1.0*	0.4*	0.5*
Pooled SEM	0.1	0.1	0.2	0.4	0.6	0.2

[1] Values are least-square means.
[2] Segment 2 is proximal, segment 7 is distal.
* Differs from infected full-fed, $P < 0.05$. ** Differs from infected full-fed, $P < 0.01$.

enterocytes on the upper villi. In the distal small intestine, lactase and alkaline phosphatase activities remained reduced at 10 d postinfection in infected-malnourished compared with infected full-fed pigs, providing further evidence that malnutrition hampers the regeneration of intestinal villi.

In conclusion, the present study provides clear evidence that feeding during the diarrheal episode is important for rapid recovery of the small intestine following rotavirus infection. The current practice of "bowel rest" might prolong the presence of diarrhea in infants infected with rotavirus. The metabolic hormone responses observed are consistent with nutrients being absorbed despite diarrhea. For example, plasma IGF-I concentrations did not decline after rotavirus infection in infected full-fed pigs compared with noninfected pigs. In addition, small intestine histology and enzymatic activities at 9 d postinfection remained reduced in infected full-fed pigs, whereas diarrhea had subsided. Together, these data indicate that other mechanisms, in addition to malabsorption, may contribute to rotaviral diarrhea. Further identification of molecular and cellular indices of rotaviral enteritis affected by PEM should enhance our understanding of those mechanisms and lead to the design of nutritional strategies for rapid recovery from diarrhea in malnourished and well-nourished children.

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