Malnutrition Modifies Pig Small Intestinal Inflammatory Responses to Rotavirus

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ABSTRACT Infectious diarrheal diseases and malnutrition are major causes of child morbidity and mortality. In this study, malnutrition was superimposed on rotavirus infection in neonatal piglets to simulate the combined intestinal stress of viral enteritis in malnourished infants. Two-day-old piglets were assigned to three treatment groups as follows: 1) noninfected, fully nourished; 2) infected, fully nourished; and 3) infected, malnourished. Intestinal indices of inflammation were monitored over the subsequent 2-wk period. Intestinal damage and diarrhea were observed within 2 d of rotavirus infection and began to subside in nourished piglets by d 9 but persisted through d 16 postinfection in malnourished piglets. Rotavirus upregulated small intestinal expression of major histocompatibility complex (MHC) class I and class II genes; malnutrition intensified MHC class I gene expression and suppressed MHC class II expression. Jejunal CD4+ and CD8+ T-lymphocyte numbers were elevated for infected, nourished piglets on d 2, 9 and 16 postinfection. Malnutrition did not significantly affect the local expansion of T cell subsets in response to rotavirus. Intestinal prostaglandin E2 (PGE2) concentrations were elevated early after rotavirus infection independent of nutritional state. By d 9, PGE2 concentrations returned to baseline in infected, nourished piglets but remained elevated in malnourished piglets, corresponding to diarrhea observations. Together, the results identify intestinal indices of inflammation that are modulated by malnutrition and prompt reconsideration of current models of rotavirus pathophysiology. J. Nutr. 129: 838–843, 1999.

KEY WORDS: • rotavirus • inflammation • malnutrition • pigs • small intestine

It has now been more than two decades since rotaviruses were determined to be the most common cause of severe dehydrating diarrhea (Bishop et al. 1973, Kapikian and Chanock 1996). Extensive child morbidity and mortality continue nonetheless. In developing countries, in which nutritional status is often compromised, rotaviruses cause >850,000 deaths each year. Worldwide, rotaviruses account for >25% of diarrheal-related deaths and 6% of all deaths in children <5 y old (Matsui and Angel 1997). Considerable promise arises from recent efforts to develop an antirotavirus vaccine (Glass et al. 1996). In fact, progress with vaccine strategies exceeds our understanding of mechanisms underlying rotavirus pathophysiology. Knowledge of the molecular and cellular basis of host responsiveness to rotavirus infection remains crucial, particularly in the context of malnutrition, which exacerbates disease severity and will likely affect the efficacy of antirotavirus vaccine strategies.

Rotavirus infections are characterized by viral replication in small intestinal enterocytes (Estes 1990), with subsequent cell lysis and attendant villous blunting (Theil et al. 1978), depressed levels of mucosal disaccharidases (Bishop et al. 1973), watery diarrhea (Theil et al. 1978) and dehydration. It is generally accepted that the destruction of villous epithelial cells reduces enzymatic and absorptive capacity in the small intestine, resulting in a malabsorptive-type diarrhea (Graham et al. 1984). With a neonatal pig model, we recently identified nutritionally responsive indices of structural repair that remain operative despite epithelial damage in rotavirus-infected intestine (Zijlstra et al. 1997). Those findings bring into question the extent to which malabsorption contributes to rotaviral diarrhea and challenge the common nutritional management protocol of “bowel rest,” i.e., reduction of luminal nutrients during the diarrheal episode (American Academy of Pediatrics 1996, Lieberman 1994, Zijlstra et al. 1997). We have now extended our observations and provide evidence that rotavirus induces an attendant intestinal inflammatory response that may contribute to a secretory-type diarrhea. Also identified are molecular and biochemical indices of rotavirus infection that are responsive to malnutrition, enabling opportunities to understand how individual macronutrients contribute at the intestinal level to host clearance of rotavirus.
MATERIALS AND METHODS

Animals and experimental design. Animal protocol was approved by the University of Illinois Laboratory Animal Care Advisory Committee and followed principles established by the NIH (NRC 1985). Experimental design, diet considerations, and infection protocol were described in more detail in a previous report of other data from this study (Zijlstra et al. 1997). Two-d-old cesarean-delivered, colorum-deprived piglets (n = 39) were randomly assigned to three treatment groups as follows: 1) noninfected, fully nourished; 2) infected, fully nourished; and 3) infected, malnourished. Pigs in groups 2 and 3 were infected with porcine rotavirus at 2 d of age.

All piglets were fed a liquid diet formulated to meet the nutritional requirements for growing piglets from birth through 3 wk of age (McClead et al. 1990) and prepared by the Mead Johnson Nutritional Group (Evansville, IN) as a dry powder. Pigs in groups 1 and 2 were fed complete reconstituted formula (180 g/L) and were pair-fed according to daily intake of the infected, fully nourished group. For the malnourished, infected group, formula was diluted 50% with water plus electrolytes and also supplied according to daily volume intake of infected, fully nourished piglets (group 2) (Buttner et al. 1985, Zijlstra et al. 1997).

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 piglets per treatment were killed by electrocution followed by exsanguination. The small intestine was dissected free of mesentery and arranged in six parts of equal length to enable collection of tissue at seven equidistant sites from the duodenum (segment 1) and proximal jejunum (segment 2) to distal jejunum (segment 6) and distal ileum (segment 7). Small intestine tissue was collected immediately and fixed for immunocytochemistry or frozen at −80°C for other analyses as described below.

Intestinal MHC class I and II expression. Intestinal major histocompatibility complex (MHC)* RNA expression was monitored as a general barometer of inflammation (Abbas et al. 1991). Total RNA was extracted from frozen jejunal tissue (segment 6) using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). Total RNA samples (10 μg each) were size-separated in 1.25% agarose/3% formaldehyde gels and immobilized onto nylon membranes (Magna Graph, Westborough, MA) by using standard Northern blotting techniques (Sambrook et al. 1989). Blots were probed with α32P-labeled cDNA probes specific for porcine MHC class I and II genes (kindly provided by Dr. L. B. Schook, Carlsbad, CA). To normalize jejunal PGE2, total protein concentrations in the methanol-insoluble fraction were determined by the modified Lowry method (Hartree 1972) with bovine serum albumin as a standard.

Statistical analyses. Data were analyzed using the General Linear Models procedure of the SAS statistical package (SAS 1985). Infected, fully nourished piglets (group 2: d 2, d 5; d 9, n = 5; d 16, n = 4) were compared with preplanned contrasts with either noninfected piglets (group 1: d 2, n = 4; d 9, n = 4; d 16, n = 4) to determine the effect of rotavirus infection, or to infected, malnourished piglets (group 3: d 2, n = 4; d 9, n = 5; d 16, n = 4) to determine the effect of malnutrition within infected piglets (Steel and Torrie 1980). 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. To consider possible mechanisms underlying intestinal responses to rotavirus alone, or to the combined effects of rotavirus and malnutrition, relationships among diarrhea observations and inflammatory variables were evaluated by Spearman correlation analysis (SAS 1985).

RESULTS

Animal observations. Nutrient intake and weight gain data were presented in a previous report of this study that focused on animal growth and metabolic and small intestinal parameters of recovery from rotavirus (Zijlstra et al. 1997). Diarrhea was not observed in any treatment group before inoculation with rotavirus (Fig. 1). Rotavirus infection resulted in severe, watery diarrhea within 24 h, which lasted for 1 wk for all infected piglets, regardless of nutritional regimen (Fig. 1). By 8 d postinfection, diarrhea began to subside in both nourished and malnourished piglets. Diarrhea cleared completely by 10 d postinfection in infected, nourished piglets (Fig. 1). In contrast, diarrhea continued through 16 d postinfection for infected, malnourished pigs, although the physical appearance of excreted material changed from a liquid consistency to a more paste-like, but easy flowing consistency (Fig. 1).

Corresponding to diarrhea observations, rotavirus infection resulted in weight loss in both nourished and malnourished, infected piglets during the initial 48 h postinfection (Zijlstra et al. 1997). Nourished, infected piglets began to regain body weight by 5 d postinfection, with rate of body weight gain similar to that of noninfected pigs at 11 d postinfection (Zijlstra et al. 1997). In contrast, after the initial 48-h period of weight loss, body weights of infected, malnourished pigs remained essentially static throughout the postinfection period.

Intestinal MHC class I and II RNA expression. At 2 d postinfection, the level of MHC class I RNA expression doubled in the distal jejunum of rotavirus-infected, nourished piglets relative to noninfected piglets (Fig. 2; P < 0.01). A similar increase in intestinal MHC class I RNA expression was observed for rotavirus-infected, malnourished piglets (Fig. 2; P < 0.1 vs. infected, nourished). At 9 d postinfection, MHC
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class I RNA expression increased further for both nutritional regimens. The level of MHC class I RNA expression in the jejenum of infected, nourished piglets was three times that observed for noninfected pigs (Fig. 2). Rotavirus infection at 2 d of age (0, no diarrhea; 1, stiff flowing feces; 2, easy flowing feces; 3, severe, watery diarrhea). Values are least-square means (n = 4–5); pooled SEM per day is symbolized as a single error bar: * infected, malnourished differs from infected, nourished, P < 0.05; ** infected, malnourished differs from infected, nourished, P < 0.01; *** infected, malnourished differs from infected, nourished, P < 0.001; **** noninfected differs from infected, nourished, P < 0.001.

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To gain additional perspective on the activation of MHC class I vs. class II gene expression in response to both rotavirus and superimposed malnutrition, the ratios of intestinal expression of MHC class I to class II were calculated. In response to rotavirus, the MHC class I/II expression ratio increased on d 2 and 9 postinfection for both nutritional regimens. The intestinal MHC class I/II ratios did not differ between noninfected and infected, nourished animals on d 16 postinfection. Because the level of MHC class I expression generally increased in response to malnutrition and the level of MHC class II was generally suppressed by malnutrition, the MHC class I/II ratio was numerically greater for infected, malnourished piglets relative to infected, nourished piglets throughout the study.

Intestinal CD4+ and CD8+ T lymphocytes. The number of jejunal CD4+ T lymphocytes in infected, nourished piglets was more than twice that of noninfected piglets on d 2, three times greater on d 9 (P < 0.05), and approximately doubled on d 16 (Fig. 3). Generally, malnutrition did not affect the temporal pattern of rotavirus-induced CD4+ T-lymphocyte expansion (Fig. 3).

Jejunal CD8+ T-lymphocyte numbers doubled in response to rotavirus on d 2 (P < 0.05; Fig. 3). A numerical (P > 0.1) increase in jejunal CD8+ T-lymphocyte numbers was also observed on d 9 and d 16 postinfection (Fig. 3). Similar to observations for CD4+ T lymphocytes, malnutrition generally did not affect the number of CD8+ T cells in the jejenum of rotavirus-infected piglets (Fig. 3). The ratio of CD4+ to CD8+ T lymphocytes was similar on each of the days examined postinfection.

Intestinal PGE2. Rotavirus infection increased PGE2 concentrations 10-fold in the mid-jejunum of nourished piglets by 2 d postinfection (P < 0.05; Fig. 4). A sixfold increase in jejunal PGE2 concentrations was observed for infected, malnourished piglets (Fig. 4). At 9 d postinfection, PGE2 concentrations in infected, nourished piglets were not different from noninfected animals and thus had returned to baseline. In contrast, jejunal PGE2 concentrations in infected, malnourished piglets remained elevated relative to infected, nourished piglets (P < 0.05; Fig. 4). Intestinal PGE2 concentrations tended to be elevated in infected, nourished piglets relative to noninfected piglets again on d 16 postinfection (P < 0.10; Fig. 4). Intestinal PGE2 concentrations remained elevated for infected, malnourished piglets on d 16, with concentrations similar to those of the infected, nourished group (Fig. 4).

Correlation analysis. Diarrhea observations were positively related to CD4+ T-lymphocyte numbers (r = 0.31; P < 0.10), CD8+ T-lymphocyte numbers (r = 0.46; P < 0.01), MHC class I expression (r = 0.53; P < 0.01) and PGE2 concentrations (r = 0.53; P < 0.001) (Table 1). The number of jejunal CD4+ T lymphocytes was positively correlated with CD8+ T-lymphocyte numbers (r = 0.42; P < 0.01), MHC class I expression (r = 0.32; P < 0.10) and PGE2 concentrations (r = 0.33; P < 0.05). The number of jejunal CD8+ T lymphocytes was positively correlated with MHC class I expression (r = 0.58; P < 0.001) and PGE2 concentrations (r = 0.39; P < 0.05). Jejunal MHC class I expression was positively correlated with MHC class II expression (r = 0.33; P < 0.10) and PGE2 concentrations (r = 0.48; P < 0.01).

DISCUSSION

Rotavirus infection of the small intestine of neonatal piglets rapidly activated local cellular and molecular components known to mediate both inflammation and immunity. Moreover, the additional insult of malnutrition prolonged inflammatory responses to rotavirus and thereby intensified and temporally extended the diarrheal episode. Convalescent dietary management of infants after acute rotavirus infection has been debated for many years (AAP 1996). This study suggests that the time-honored treatment method of “bowel rest” after rotavirus infection may delay mucosal recovery.

Rotavirus infection is characterized by viral replication in small intestinal enterocytes with subsequent cell lysis and attendant villous blunting, depressed levels of mucosal disac-
charidases, watery diarrhea and dehydration (Bishop et al. 1973, Estes 1990, Theil et al. 1978). Because of the reduced enzymatic and absorptive capacity in the small intestine, it is generally accepted that rotavirus elicits a malabsorptive-type of diarrhea (Graham 1984, Rhoads et al. 1991). On the basis of clear evidence of local T-lymphocyte expansion, enhanced intestinal MHC class I and class II gene expression and elevated tissue concentrations of PGE$_2$, we propose that intestinal inflammatory responses to rotavirus may contribute to a secretory-type of diarrhea.

In a previous report on this piglet model, we demonstrated that the onset of rotaviral diarrhea coincided with villous destruction and reduction of mucosal disaccharidase activities (Zijlstra et al. 1997). Those observations agree with reports on other experimental models of rotavirus infection and are consistent with the possibility that malabsorption contributes to rotaviral diarrhea (Zijlstra et al. 1997). However, a clear distinction between intestinal damage and diarrhea was established from the observation that diarrhea subsided by 9 d postinfection in nourished but not in malnourished piglets, whereas the degree of intestinal structure damage was comparable between these nutritional groups at that time postinfection (Zijlstra et al. 1997). Thus, it is unlikely that malabsorption resulting from epithelial damage is the sole explanation for rotaviral diarrhea.

Results from other model systems and limited observations from human studies also invoke reconsideration of the pathophysiology of rotavirus-induced diarrhea. At least three other reports on a piglet model demonstrate rotavirus-induced diarrhea before extensive damage to the intestinal epithelium (McAdaragh et al. 1980, Theil et al. 1978). For example, piglets inoculated with porcine rotavirus developed watery
diarrhea 8 h after infection, whereas minor damage to jejunal segments was not observed until 48 h postinfection (Vellenga et al. 1992). Similarly, for a mouse model, there was no correlation between the number of rotavirus infected cells and the severity of diarrhea (Bass and Greenberg 1995). Studies with a heterologous mouse model demonstrate that chemically inactivated rhesus monkey rotavirus induces diarrhea in the absence of epithelial attachment, cellular entry or viral replication (Shaw et al. 1995). It has also been reported that diarrhea preceded obvious cell damage in human infants infected with rotavirus (Bass and Greenberg 1995). Among intestinal biopsy specimens from 40 rotavirus-infected infants, only 5% exhibited histologic evidence of damage (Kohler et al. 1990).

New concepts of rotaviral pathogenesis are evoked most convincingly from the recent identification of a rotaviral enterotoxin (Ball et al. 1996, Dong et al. 1997, Glass et al. 1996). While making an antiserum to the nonstructural rotaviral glycoprotein, NSP4, Estes and co-workers fortuitously discovered that intraperitoneal delivery of purified NSP4 induced diarrhea in a mouse model (Ball et al. 1996). Subsequent studies from this group with several model systems clearly demonstrate that NSP4 alters Ca2+ homeostasis in host cells through receptor-mediated phospholipase C activation and inositol 1,4,5-triphosphate production (Dong et al. 1997). That finding is consistent with numerous other examples of the mediation of infectious diarrhea through altered intracellular Ca2+ homeostasis, leading to Ca2+-dependent fluid secretion across the mucosa toward the lumen (Argenzio 1996). Local prostaglandins can also serve as mediators of intestinal ion imbalances, resulting in epithelial chloride secretion across the mucosa toward the lumen (Kandil et al. 1994). The possible contributions of PGE2 to rotaviral and specifically NSP4-induced diarrhea should be defined, considering the present evidence that malnutrition exacerbates PGE2 responses to rotavirus, together with clear evidence that tissue PGE2 concentrations can be altered.

### TABLE 1

<table>
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<th>Diarrhea</th>
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<th>MHCII</th>
<th>PGE2</th>
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<td>0.58***</td>
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<td>x</td>
</tr>
<tr>
<td>PGE2</td>
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<td>0.33†</td>
<td>0.39†</td>
<td>0.48**</td>
<td>0.15 †</td>
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1 Correlation coefficients (r) were calculated by using observations from the 16-d experimental period. CD4, jejunal CD4+ T-lymphocyte numbers; CD8, jejunal CD8+ T-lymphocyte numbers; MHCI, major histocompatibility complex (MHC) class I RNA expression; MHCII, MHC class II RNA expression; PGE2, jejunal prostaglandin E2 concentrations. Superscripts in body of the table indicate level of significance: †, P < 0.05; **, P < 0.01; ***; P < 0.001; †, P < 0.10.
through manipulation of diet fatty acid composition (Fritsche et al. 1993).

Prolonged diarrhea in infected, malnourished piglets was associated with more intense and prolonged expression of local mediators or markers of intestinal inflammation. Relative to infected, nourished piglets, intestinal PGE2 concentrations were greater and remained elevated longer in malnourished piglets also infected with rotavirus. Similarly, patterns of intestinal MHC class I gene expression, an acutely sensitive barometer of local inflammation, were positively and significantly correlated with the pattern of PGE2 expression and with prolonged diarrhea. Those results are consistent with substantial evidence that undernutrition typically intensifies viral enteritis and results in greater morbidity and mortality from infections (Cunningham-Rundles 1994). The identification of specific indices of rotavirus infection that are responsive to malnutrition enables further studies to determine how individual macronutrients contribute at the intestinal level to host clearance of rotavirus. That information may facilitate the design of rational nutritional therapies to enhance recovery from rotavirus infection, or to maximize responses to rotavirus immunization protocols in geographic regions subject to malnutrition.

LITERATURE CITED


