Alanyl-glutamine promotes intestinal epithelial cell homeostasis in vitro and in a murine model of weanling undernutrition

Priscilla M. Ueno,1 Reinaldo B. Oriá,2 Elizabeth A. Maier,1 Marjorie Guedes,3 Orlie Conde,2 David Wu,4 Tara Willson, Simon P. Hogan,4 Aldo A. M. Lima,3 Richard L. Guerrant, D. Brent Polk,6 Lee A. Denson,3 and Sean R. Moore1

1Division of Gastroenterology, Hepatology, and Nutrition, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio; 2Department of Pathology and 1Institute of Biomedicine, Federal University of Ceará, Fortaleza, Brazil; 3Division of Allergy and Immunology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio; 5Center for Global Health, University of Virginia, Charlottesville, Virginia; and 6Department of Pediatrics, University of Southern California, Los Angeles, California

Submitted 29 November 2010; accepted in final form 24 July 2011

Ueno PM, Oriá RB, Maier EA, Guedes M, de Azevedo OG, Wu D, Willson T, Hogan SP, Lima AA, Guerrant RL, Polk DB, Denson LA, Moore SR. Alanyl-glutamine promotes intestinal epithelial cell homeostasis in vitro and in a murine model of weanling undernutrition. Am J Physiol Gastrointest Liver Physiol 301: G612–G622, 2011. First published July 28, 2011; doi:10.1152/ajpgi.00531.2010.—Alanyl-glutamine (Ala-Gln) has recently been shown to enhance catch-up growth and gut integrity in undernourished children from Northeast Brazil. We hypothesized that the intestinal epithelial effects of Ala-Gln in malnourished weanling mice and mouse small intestinal epithelial (MSIE) cells would include modulation of barrier function, proliferation, and apoptosis. Dans of 10-day-old suckling C57BL/6 pups were randomized to a standard diet or an isocaloric Northeast Brazil “regional basic diet,” moderately deficient in protein, fat, and minerals. Upon weaning their dam’s diet on day of life 21, pups were randomized to Ala-Gln solution or water. At 6 wk of age, mice were killed, and jejunal tissue was collected for morphology, immunohistochemistry, and Ussing chamber analysis of transmucosal resistance and permeability. Proliferation of MSIE in the presence or absence of Ala-Gln was measured by MTS and bromodeoxyuridine assays. MSIE apoptosis was assessed by annexin and 7-aminoactinomycin D staining. Pups of regional basic diet-fed dams exhibited failure to thrive. Jejunal specimens from undernourished weanlings showed decreased villous height and crypt depth, decreased transmucosal resistance, increased permeability to FITC-dextran, increased claudin-3 expression, and decreased epithelial proliferation and increased epithelial apoptosis (as measured by bromodeoxyuridine and cleaved caspase-3 staining, respectively). Undernourished weanlings supplemented with Ala-Gln showed improvements in weight velocity, villous height, crypt depth, transmucosal resistance, and epithelial proliferation/apoptosis compared with unsupplemented controls. Similarly, Ala-Gln increased proliferation and reduced apoptosis in MSIE cells. In summary, Ala-Gln promotes intestinal epithelial homeostasis in a mouse model of malnutrition-associated enteropathy, mimicking key features of the human disease.

1-αlanyl-1-glutamine; glutamine dipeptide; protein malnutrition; tropical enteropathy; environmental enteropathy; claudin-3

Undernutrition is a leading cause of child morbidity and mortality in developing countries, where an estimated 32% of preschool children are stunted, 20% are underweight, and over one-half of all child deaths are linked to malnutrition (4, 10). Malnutrition-associated enteropathy, also known as tropical or environmental enteropathy, is a subclinical disorder of malabsorption and increased intestinal permeability (33, 39). It is highly prevalent in the developing world and postulated to result from the combined effects of marginal diets, unsanitary environments, and repeated and persistent enteric infections (both symptomatic and asymptomatic) (35, 39). It is further hypothesized to contribute to a reciprocal cycle of childhood malnutrition and infection and to mitigate the efficacy of oral vaccines against gut or gut-acquired pathogens (32, 39, 47, 61). The typical pathological features of malnutrition enteropathy include small bowel villous atrophy, crypt hyperplasia, and lamina propria lymphoplasmacytic inflammation; however, the pathogenesis of these changes is only partially understood, as are optimal approaches to prevention and therapy (6, 24, 28, 34, 39, 43, 45, 60, 66).

Several randomized, controlled trials have examined the extent to which the amino acid glutamine (41, 69), or its more stable and soluble dipeptide alanyl-glutamine (Ala-Gln) (44), are efficacious “repair nutrients” to restore intestinal barrier function and promote growth in children with malnutrition-associated enteropathy. Glutamine, the most abundant free amino acid in plasma, is considered conditionally essential for gut homeostasis during severe catabolic states (73). Furthermore, glutamine is known to activate a number of proliferative and cytoprotective signals that mediate wound repair, and improve intestinal permeability. The first trial of glutamine for malnutrition enteropathy showed that supplementation improved intestinal barrier function, but did not enhance catch-up growth in underweight children from Northeast Brazil (41). A second trial, in Gambian infants, found that lower doses of glutamine did not prevent growth faltering, nor promote intestinal barrier function (69). Most recently, Ala-Gln supplementation was shown to improve both weight gain and paracellular intestinal permeability in mild-to-moderately undernourished children from Northeast Brazil (44).

Positive results from the Ala-Gln study prompted us to further elucidate the mechanisms by which Ala-Gln ameliorates malnutrition-associated enteropathy. We designed experiments in a modified murine model of weanling malnutrition and in intestinal epithelial cell culture to test the following hypotheses: 1) Ala-Gln protects against enteropathy in weanling mice fed an established “regional basic diet” (RBD), moderately deficient in protein, fat, and minerals (formulated to mimic the typical diet of poor populations in Northeast Brazil) (65), and 2) Ala-Gln modulates intestinal epithelial cell...
proliferation and apoptosis in undernourished mice and in vitro. We show herein that this mouse model of weanling malnutrition phenocopies key features of malnutrition-associated enteropathy in humans and further demonstrate that Ala-Gln attenuates the mucosal atrophy, barrier defects, and alterations in intestinal epithelial proliferation and apoptosis induced by malnutrition.

MATERIALS AND METHODS

Murine model of undernutrition. Wild-type C57BL/6 mice were purchased from the Comprehensive Cancer Core at Cincinnati Children’s Hospital Medical Center (Cincinnati, OH) and housed in a barrier facility with an ambient temperature of 22°C, a relative humidity ranging from 30 to 70%, and a 14:10-h light-dark cycle. Breeding pairs were maintained in the same cage until females were visibly pregnant. Females were then placed in separate cages with free access to a standard diet and water and monitored daily for delivery. When pups reached day of life 10, dams were randomized to one of two groups: group 1 dams were given a balanced control diet, and dams in group 2 were given an isocaloric RBD (Fig. 1, Table 1). The RBD is a well-studied rodent diet high in carbohydrates and marginally deficient in protein, fat, and minerals. It is formulated to represent the multideficient diets of poor populations in Northeast Brazil (65), where protein-energy malnutrition remains a serious public health problem, and where Gln and Ala-Gln have both been shown to improve gut integrity in undernourished children (41, 44). Both the RBD and control diets were administered ad libitum as purified diets (Research Diets, New Brunswick, NJ), supplemented with vitamins and irradiated before administration. Attempts to introduce the RBD on day of life 1 and day of life 3 led to the loss of several pups. We then revised our protocol to delay introduction of the diets until day of life 10, with no further loss of pups.

Before weaning, dams and pups were weighed thrice weekly. On day of life 21, pups were weaned to their dams’ diet, i.e., group 1 pups were weaned to the control diet, and group 2 pups were weaned to the RBD. Upon weaning, pups in both groups were randomized to one of two subgroups with free access to either plain drinking water or a solution of 111 mM of L-alanyl-L-glutamine (Ajinomoto Amino-Science, Raleigh, NC) in water. Weanlings were caged together (up to 4 per cage), according to their experimental group: 1) nourished, untreated; 2) nourished, Ala-Gln treated; 3) undernourished, untreated; and 4) undernourished, Ala-Gln treated. An oral route of administration was chosen to mimic the conditions under which Ala-Gln has been studied in undernourished children (44). Ad libitum administration in the drinking water was selected following technical difficulties in administering daily oral gavages over several weeks in underweight mice. We measured the body weight and tail length of each mouse on a weekly basis, as well as the collective consumption of food and water by cages of mice in their respective experimental groups. We killed mice at 6 wk of age by CO2 inhalation and cervical dislocation. Mice were allowed access to food and water (± Ala-Gln)

![Diagram of murine model of weanling undernutrition.](image)

**Outcomes:**
- Weight gain/tail length
- Intestinal barrier function
- Jejunal morphology

**Table 1. Composition of the multideficient regional basic diet and control diet**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control Diet</th>
<th>Regional Basic Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Corn starch</td>
<td>346</td>
<td>1,384</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>45</td>
<td>180</td>
</tr>
<tr>
<td>Dextrose</td>
<td>250</td>
<td>1,000</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>2.41</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Inulin</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>630</td>
</tr>
<tr>
<td>Mineral mix S10026</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mineral mix S10026A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>5.5</td>
<td>0</td>
</tr>
<tr>
<td>Potassium citrate, 1 H2O</td>
<td>16.5</td>
<td>0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0</td>
<td>1.9</td>
</tr>
<tr>
<td>Vitamin mix V10001</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

**Total**
1,071.05 4,071 1,112.93 4,081
until the morning of death. In a subset of 13 treated vs. untreated undernourished mice, we obtained a single blood sample by cardiac puncture immediately following death using pentobarbital sodium injection. We analyzed plasma glutamine levels using a Hitachi automated amino acid analyzer. All mouse protocols were approved by, and performed in accordance with, the Institutional Animal Care and Use Committee of the Cincinnati Children’s Hospital Medical Center.

Ex vivo intestinal barrier function. We measured intestinal permeability using Ussing chambers. Following death, mid-jejunal segments were excised and flushed with PBS. Each segment was opened along the mesenteric border, rinsed, and mounted in Ussing diffusion chambers with an exposure area of 0.30 cm² (U-2500 Dual-Channel Ussing chamber, Warner Instruments, Hamden, CT). The mucosal sides were excised and flushed with PBS. Each segment was opened along

Intestinal morphometry. Jejunal segments were sectioned longitudinally, fixed in 4% formalin, embedded in paraffin, and stained with routine hematoxylin-eosin. Intestinal villus and crypt dimensions were measured in a blinded manner according to Coutinho et al. (14).

Immunohistochemistry. Jejunal samples were sectioned at 5 μm, placed in 10 mM citrate buffer of pH 6.0, and heated for 10 min. Sections were incubated for 15 min in 3% hydrogen peroxide (Sigma-Aldrich) in methanol, washed with distilled water and phosphate-buffered saline for 5 min each, permeabilized in 0.3% Triton (Sigma-Aldrich) for 15 min and in 0.1% Tween (Fisher Scientific, Pittsburgh, MA) for 5 min, blocked in 10% normal goat serum for 1 h at room temperature (RT), and then incubated with primary antibody-cleaved caspase-3 (Asp175) (Cell Signaling, Boston, MA) overnight at 4°C. After washing, sections were incubated with conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at RT, then permeabilized twice with 0.1% Tween for 5 min, washed, and incubated for 1 h at RT with R.T.U. Vectastain Elite ABC Reagent, according to the manufacturer’s protocol (Vector Laboratories). After washing, the section was developed with diaminobenzidine substrate kit, 3,3’-diaminobenzidine (Vector Laboratories), to give a brown to gray/black color. Slides were dehydrated in serial ethanol and xylene solution and permanently mounted. Images were digitally captured at ×100 using an Olympus BX51 microscope, and quantification of cleaved caspase-3 staining was performed in a blinded manner by counting positive cells in multiple random microscope fields per tissue section.

For bromodeoxyuridine (BrdU) staining, mice were injected intraperitoneally with 1.0 ml concentrated reagent/100 g body wt (Zymed Laboratories, Invitrogen Immunodetection) and then killed 2 h after injection. Jejunal tissue was fixed and embedded in paraffin, as described above. Sections were stained per the manufacturer’s protocol (BrdU kit; Invitrogen). Images were digitally captured at ×100 using an Olympus BX51 microscope, and quantification of BrdU staining was performed in a blinded manner by counting positive cells in multiple random microscope fields per tissue section.

Fluorescent immunohistochemistry. Jejunal sections were flushed with cold PBS and fixed for 4–6 h in 4% paraformaldehyde/1 × PBS at 4°C. Tissues were transferred to 30% sucrose/PBS overnight at 4°C and embedded in O.C.T. Tissue-Tek. Frozen tissues were sectioned at 5 μm, heated 15 min at 40°C, cooled to RT, dehydrated in methanol, and fixed in acetone at −20°C. Slides were rinsed in PBS and then submerged in blocking serum containing PBS/2% BSA/4% goat serum for 2 h at RT. Blocking serum was removed, and slides were rinsed with PBS, then covered with primary antibody diluted in blocking serum [rabbit anti-claudin-1 (Abcam) 5 μg/ml; rabbit anti-claudin-3 (Invitrogen) 1.25 μg/ml; rabbit anti-occludin (Invitrogen) 0.25 μg/ml; rabbit anti-zonula occludens-1 (ZO-1) (Invitrogen) 0.25 μg/ml], and incubated overnight at 4°C in a humidified chamber. Slides were rinsed at RT in PBS and then rinsed in wash buffer (0.1% BSA/0.5% Tween 20/PBS) and again in PBS. All subsequent steps were performed in the dark in a humidified chamber. Sections were covered with secondary antibody [goat anti-rabbit IgG AF488 (Invitrogen) 8 μg/ml] at RT for 2 h. Slides were then washed as before, covered with Fluoromount/DAPI, and stored in the dark at 4°C until photographed (within 48 h of completion).

Western blot and densitometry. Jejunal segments were lysed in RIPA buffer using the Mammalian Cell Lysis Kit (Sigma). Supernatants were quantified by the Bradford method (Bio-Rad), and 40 μg were separated and transferred to a nitrocellulose membrane using the Novex NuPage Bis-Tris Electrophoresis System (Invitrogen), according to the manufacturer’s instructions. The membrane was washed in wash buffer (1 × Tris-buffered saline + 0.1% Tween 20), then blocked for 1 h in 0.5% nonfat dry milk (BioRad)/wash buffer, and then probed with 1° antibody in 0.5% block, for either 1 h at RT [monoclonal anti-B-actin (Sigma)] or overnight at 4°C [rabbit anti-claudin-3 (Invitrogen)]. The membrane was then washed in wash buffer and then probed with the appropriate horseradish peroxidase (HRP) conjugate 2° antibody [anti-Ms or anti-Rb IgG, HRP-linked (Cell Signaling)] for 1 h at RT. The HRP signal was detected by chemiluminescence using the SuperSignal West Pico Kit (Thermo Scientific) and autography (Amersham). Densitometric analyses were performed with ImageJ software (National Institutes of Health).

Cell culture. Mouse small intestine epithelial (MSIE) cells (a gift from Dr. Robert Whitehead, Vanderbilt University) were used for all in vitro experiments. This nontransformed, conditionally immortalized cell line was chosen to maintain species consistency between in vivo and in vitro experiments. Before plating, dishes were precoated with collagen [100 μg collagen I (BD Bioscience, Franklin Lakes, NJ) in 2.3 ml of glacial acetic acid and 2.0 liters of distilled water], and washed twice with sterile PBS. Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 2 mM glutamine, 5% fetal bovine serum, 0.1% insulin-transferrin-selenium-A, 1% penicillin-streptomycin (50 U of penicillin/ml, and 50 μg of streptomycin/ml, all from Invitrogen), and 25 μl of recombinant mouse IFN-γ (R&D Systems, Minneapolis, MN) at the permissive temperature of 33°C in 5% CO2. For experiments, cells were maintained in glutamine-free and serum-free RPMI 1640 in the presence or absence of Ala-Gln (1.0 mM) for 18 h at 37°C in 5% CO2. This temperature reverts MSIE cells to a quiescent, wild-type state (48). No other amino acids were substituted for glutamine as a control for Ala-Gln.

BrdU cell proliferation assay. Cell proliferation was determined by a colorimetric BrdU Cell Proliferation Assay kit (Chemicon International/Millipore, Temecula, CA) per manufacturer’s instructions. MSIE cells were seeded on a 96-well flat bottom culture plates (100 μl, 5,000 cells/well; 6 wells/treatment) and incubated at 33°C (permissive) for 24 h in RPMI medium (2 mM glutamine, 5% fetal bovine serum; 0.1% insulin-transferrin-selenium-A; 1% penicillin/streptomycin; 2 mM glutamine (Invitrogen, Grand Island, NY)). Following adherence, cells were incubated at 37°C (nonpermissive) for 18 h and maintained in the following RPMI media conditions: glutamine free, serum free, and 1 mM Ala-Gln; glutamine free, serum free, and Ala-Gln null.

MTS cell viability assay. Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Cell Titer 96 AQueous, One Solution Cell Proliferation Assay, Promega, Madison, WI), which measures the activity of enzymes that reduce MTS to formazan. MSIE cells were plated in 96-well plates (100 μl, 5,000 cells/well; 6 wells/treatment) and incubated at 33°C for 24 h. Cells were then maintained in glutamine-free and serum-free media for 18 h at 37°C and analyzed per the manufacturer’s protocol.
Annexin V-FITC apoptosis and 7-amino-actinomycin D cell death assays. MSIE cells were maintained in growth media overnight at 33°C and 5% CO2 until 80% confluence. The following day, cells were starved in identical conditions to the MTS experiments described above. For flow cytometry experiments, cells were washed in PBS with 1% BSA, then resuspended in sterile water with annexin V binding buffer (BD Bioscience, San Jose, CA), then double stained with FITC annexin V and 7-amino-actinomycin D (BD Bioscience) to identify apoptotic and nonviable cells, respectively. Cells were analyzed on a FACSDIVA (Canto, Becton Dickinson, Franklin Lake, NJ) instruments software, and proportions of viable vs. nonviable and early vs. late apoptotic cells were measured, as described by Larson et al. (38).

Statistical analysis. Statistical tests were performed using GraphPad Prism (version 5.0b, GraphPad Prism, La Jolla, CA). Values are expressed as means ± SE, and statistical significance was determined by Mann-Whitney U, Kruskal-Wallis, and one-way or repeated-measures ANOVA tests, with corrections for multiple comparisons as appropriate. P values < 0.05 were considered statistically significant. All in vitro and in vivo experiments were performed at least three times.

Fig. 2. A: Ala-Gln accelerates catch-up growth in undernourished weanling mice. Pups of dams fed the regional diet (n = 14) exhibited significant failure to thrive (*P < 0.001, repeated-measures ANOVA) compared with pups of dams fed the control diet (n = 13). B: Ala-Gln treatment had no significant effect on overall weight gain in mice receiving either diet (n = 6–7 mice per group). C: however, from age 5–6 wk, weight velocity (grams per day of weight gain) was significantly greater in Ala-Gln-treated malnourished mice compared with the other 3 groups. *P < 0.05, Kruskal-Wallis test with Dunn’s multiple-comparisons test. Values are means ± SE.

Fig. 3. Effects of malnutrition and Ala-Gln on intestinal barrier function. A: measurements of transmucosal resistance in Ussing chamber-mounted jejunal segments revealed significant differences between untreated, well-nourished and malnourished mice, as well as differences between malnourished mice treated with Ala-Gln vs. water. *P < 0.05, ANOVA with Bonferroni correction for multiple comparisons. Values are means ± SE; n = 8–18 mice per group. B: no significant (NS) differences in intestinal short-circuit current (Isc) were detected. C: untreated, malnourished mice showed increased transmucosal permeability to 4-kDa FITC-dextran compared with untreated well-nourished controls. *P < 0.01, ANOVA with Bonferroni correction. Ala-Gln treatment of well-nourished and undernourished mice produced a modest increase and decrease, respectively, in jejunal permeability to FITC-dextran relative to untreated controls; however, neither effect was statistically significant.
RESULTS

Ala-Gln accelerates catch-up growth in undernourished weanling mice. Dams of 10-day-old suckling pups were randomized to one of two diets (Table 1, Fig. 1). Group 1 dams received a balanced control diet (carbohydrates 63%, protein 19%, fat 7%), and group 2 dams received an isocaloric, multideficient Northeast Brazil RBD (carbohydrates 83%, protein 6%, fat 2%). RBD-fed dams consumed less food than control dams (9.7 vs. 6.4 g of feed/day; \( P < 0.05 \), unpaired \( t \)-test) and lost weight more quickly (0.86 vs. 0.46 g/day; \( P < 0.05 \), repeated-measures ANOVA). Consumption of drinking water did not differ significantly between groups. As shown by the body weight measurements in Fig. 2A, suckling pups of RBD-fed dams exhibited significant failure to thrive compared with controls (\( P < 0.001 \), repeated-measures ANOVA). The mean tail length of pups of RBD-fed dams increased at one-half the rate of controls (0.9 vs. 1.8 mm/day; \( P < 0.001 \)).

Pups were weaned on \textit{day of life 21} to their dam’s diet and maintained on this diet until 6 wk of age. RBD-fed weanlings remained underweight and stunted relative to nourished controls until death (Fig. 2B). Ala-Gln supplementation had no significant effect on overall weight gain or tail length from age 3–6 wk; however, undernourished weanlings treated with Ala-Gln showed markedly improved weight velocity from age 5–6 wk vs. controls (Fig. 2C; \( P < 0.05 \), Kruskal-Wallis test with Dunn’s multiple-comparison tests). Weekly estimates of weight-corrected food and water intake postweaning showed no significant effect of Ala-Gln on feed efficiency or water consumption in nourished or undernourished mice. To evaluate potential factors accounting for increased weight velocity in Ala-Gln-treated weanlings, we killed mice at 6 wk of age to compare intestinal barrier function and morphology.

Effects of Ala-Gln on intestinal barrier function and mucosal atrophy in undernourished mice. To determine RBD and Ala-Gln effects on intestinal barrier function, we measured transmucosal resistance (TER), \( I_{sc} \), and cumulative flux of 4-kDa FITC-dextran across ex vivo jejunal segments mounted in Ussing chambers. As shown in Fig. 3A, untreated RBD-fed mice demonstrated significantly reduced TER compared with either nourished controls or undernourished mice supplemented with Ala-Gln (\( P < 0.01 \), repeated-measures ANOVA with Bonferroni correction for multiple comparisons). We detected no significant differences in \( I_{sc} \) between experimental groups (Fig. 3B); however, RBD-fed mice exhibited a greater than twofold increase in flux of FITC-dextran compared with untreated well-nourished controls (Fig. 3C, \( P < 0.05 \), Kruskal-Wallis with Dunn’s correction for multiple comparisons). Ala-Gln treatment of well-nourished and undernourished mice produced modest increases and decreases, respectively, in jejunal permeability to FITC-dextran relative to their untreated controls; however, neither effect was statistically significant.

To evaluate potential molecular mechanisms to account for the increase in paracellular permeability we observed in untreated undernourished mice, we analyzed jejunal specimens for the expression and localization of tight junction proteins ZO-1, occludin, claudin-1, and claudin-3. Figure 4A shows representative immunofluorescent staining for each of these proteins in untreated, undernourished mice and well-nourished controls. Expression and localization of ZO-1, occludin, and claudin-1 appeared similar between groups. In contrast, claudin-3 expression was consistently elevated in the crypts of jejunal samples from undernourished mice vs. controls. Likewise, Western blotting of jejunal lysates showed a significant increase in claudin-3 expression in undernourished mice (Fig. 4, B and C).

Morphometric analyses of jejunal villous-crypt architecture revealed marked decreases in villous height, crypt depth, and villous height-to-crypt depth ratios in untreated RBD-fed mice vs. nourished controls and Ala-Gln-treated RBD-fed mice (Fig. 5). Mean peripheral plasma levels of glutamine in treated vs. untreated, undernourished mice did not differ significantly (575.3 and 513.6 μmol/l, respectively, \( P = 0.36 \)); however,
both levels were substantially below a recently reported mean value of 800 μmol/l for wild-type, well-nourished C57BL/6 mice (50).

Ala-Gln increases intestinal epithelial proliferation and decreases intestinal epithelial apoptosis in undernourished mice. Based on previous in vitro studies, we hypothesized that in vivo effects of Ala-Gln on villous height or gut integrity might result from decreased epithelial apoptosis and increased proliferation. We stained jejunal tissue for BrdU (Fig. 6A) and cleaved caspase-3 (Fig. 6C), specific markers of proliferation and apoptosis, respectively. Quantification of BrdU immunostaining revealed a greater number of proliferative epithelial cells in untreated, nourished vs. untreated, undernourished mice (Fig. 5B, P < 0.05, Kruskal-Wallis test with Dunn’s correction for multiple comparisons). Furthermore, BrdU-positive cells were seen more frequently in RBD-fed mice randomized to Ala-Gln vs. water (P < 0.05). In contrast, quantification of cleaved caspase-3 staining showed higher numbers of positively stained cells in untreated RBD-fed mice vs. control mice (Fig. 5D, P < 0.05). Moreover, RBD-fed mice randomized to Ala-Gln had significantly fewer caspase-positive cells compared with untreated, undernourished controls (P < 0.05). Ala-Gln supplementation of well-nourished mice had no effect on epithelial apoptosis; however, we did note a modest but consistent decrease in BrdU-positive cells in this group (P = 0.09).

Ala-Gln increases proliferation and survival of cultured mouse small intestinal epithelial cells. To further assess the effects of Ala-Gln on intestinal epithelial cell homeostasis, we performed viability, proliferation, and apoptosis assays with serum-starved MSIE cells maintained in the presence or absence of 1.0 mmol/l Ala-Gln. Following 18 h of treatment, we observed that Ala-Gln promoted increased viability of treated vs. untreated MSIE cells, as measured by MTS assay (P < 0.05, Fig. 7A) and increased proliferation as measured by BrdU assay (P < 0.05, Fig. 7B). To determine the role of apoptosis in Ala-Gln-mediated cell viability, we stained cells with FITC-annexin V and 7-amino-actinomycin D, then analyzed cells by flow cytometry. Figure 7, C and D, shows that Ala-Gln treatment protected MSIE cells from apoptosis (5.7 vs. 10.3% total

Fig. 5. Ala-Gln protects normal villus-crypt intestinal architecture in malnourished weanling mice. A: jejunal histology (hematoxylin-eosin ×200) in nourished (control diet) vs. malnourished (regional diet) 6-wk-old mice treated or untreated with oral Ala-Gln. Morphometric analyses of villus height (B), crypt depth (C), and villus height-to-crypt depth ratio (D) are shown. *P < 0.001 for untreated malnourished mice vs. all other groups by ANOVA with Bonferroni correction. Values are means ± SE; n = 6 to 7 mice per group.
apoptosis in supplemented vs. unsupplemented cells, $P < 0.05$), which was primarily attributable to a twofold reduction in early apoptosis.

**DISCUSSION**

This study demonstrates important effects of Ala-Gln in opposing malnutrition-induced defects in intestinal epithelial proliferation, apoptosis, and villous-crypt architecture. Although the specific mechanisms are not yet clear, these protective effects were associated with improved catch-up growth and TER in undernourished mice. The results also demonstrate that malnutrition secondary to a multideficient RBD leads to deranged jejunal mucosal structure and barrier function and provides evidence for a role of abnormal epithelial proliferation, apoptosis, and claudin-3 expression in these effects. Therefore, this mouse model of weanling malnutrition effectively recapitulates key features of human malnutrition-associated enteropathy and responses to Ala-Gln treatment seen in a recent randomized controlled trial of Ala-Gln in undernourished children with enteropathy (44).

Elucidating the cellular and molecular mechanisms of repair nutrients is important to develop a clear understanding of interventions for malnutrition-associated enteropathy, as well as its pathophysiology. We showed that supplementation of Ala-Gln in the drinking water of undernourished weanling mice enhanced catch-up growth and protected against enteropathy by increasing villous height, crypt depth, TER, and epithelial proliferation, as well as decreasing epithelial apoptosis relative to unsupplemented controls. In addition, Ala-Gln-treated undernourished weanlings showed a tendency toward improved paracellular integrity compared with untreated controls; however, this effect was not statistically significant. This mouse model, therefore, offers a promising approach for future studies of the cellular and molecular mechanisms by which Ala-Gln hastens catch-up growth and improves mucosal architecture and gut integrity in the setting of malnutrition, with further potential to examine effects on paracellular barrier function at different doses or lengths of treatment.

We also showed that suckling mice of dams fed the RBD (a diet moderately deficient in fat, protein, and minerals) fail to thrive, and that the small intestines of undernourished weanlings maintained on this diet exhibit partial mucosal atrophy, decreased TER, increased paracellular permeability, increased claudin-3 expression, decreased epithelial cell proliferation, and increased epithelial cell apoptosis. Initially formulated to reflect the marginal diets of impoverished populations in Northeast Brazil with protein-energy malnutrition (65), over the past two decades, the RBD has been well-studied in rat or mouse models of schistosomiasis (15, 16, 26, 51), hypertension (13), and nutritional neuropathies (1, 3, 17, 31, 57). The present...
study is the first of which we know to identify and characterize the enteropathy induced by the RBD. In addition, our study is the first to administer the RBD and control diets in a purified, irradiated form and to employ an isocaloric control. Thus the differences we observed between RBD-fed mice and controls may be reliably attributed to the overall nutritional composition of the diets, rather than differences in consistency, contaminants, or caloric density. Given that protein-energy malnutrition and food insecurity remain critical public health challenges throughout the developing world, the relevance of the RBD to studies of malnutrition enteropathy certainly extends far beyond Brazil (25).

We found that administration of the RBD to weanling mice led to a defect in gut integrity, as measured by both TER and permeability to FITC-dextran, an assay of paracellular barrier function. Transmucosal and paracellular barrier defects have previously been shown in a guinea pig model of protein malnutrition (58). To our knowledge, the finding that jejunal claudin-3 expression is enhanced in the setting of weanling undernutrition is novel and provides a potential mechanism for the barrier defects we observed. Clark et al. (12) reported increased claudin-3 expression in the small intestinal crypts of rats with experimental necrotizing enterocolitis and disrupted barrier function. More recently, Groschwitz et al. (30) demonstrated a link between dysregulated gut integrity and altered crypt expression of claudin-3 in mice deficient in mast cells. In the present study, we detected no differences in occludin, ZO-1, or claudin-1 expression or localization in undernourished mice. Li et al. (40) showed a decrease in claudin-1 expression, but no change in occludin expression, in the small intestines of neonatal rats subjected to a more acute and severe model of protein deprivation. These authors also reported that neither glutamine nor glutamate improved weight gain or increased claudin-1 expression. In contrast, we determined that Ala-Gln improved weight velocity and TER in undernourished mice; however, we did not observe a significant effect of Ala-Gln on paracellular permeability. Therefore, we did not analyze tight junctions in treated mice. The significant protective effect of Ala-Gln on TER in undernourished mice, in the absence of a dramatic effect on macromolecule paracellular permeability, suggests that Ala-Gln modulates other components of epithelial transport and barrier function, as indicated by previous reports of Ala-Gln’s effects on sodium and water absorption in animal models of cryptosporidiosis and cholera (5, 42).

A significant limitation of our study is that we have not yet compared Ala-Gln’s in vivo or in vitro effects with an isonitrogenous or isoenergetic control, e.g., a mixture of other nonessential amino acids. Dialanine might provide another control for Ala-Gln and allow us to assess the relative contribution of glutamine vs. alanine in promoting intestinal homeostasis in the model. Our study was not designed to adequately quantify the effects of the RBD or Ala-Gln on food and water consumption in individual mice; however, our limited analyses detected no significant effects of the RBD or Ala-Gln on postwean intake. In contrast, we found that RBD-fed dams
consumed less food and lost weight more rapidly; hence a pair feeding design (in which controls are given an amount of food equal to that consumed by mice on the deficient diet) would have provided a more rigorous control. Nonetheless, we did observe significant effects of oral Ala-Gln supplementation on catch-up growth and intestinal health that mirror results from human studies; thus these findings merit further consideration regarding potential mechanisms.

Tightly coordinated epithelial proliferation and apoptosis are essential features of healthy intestinal crypt-villous architecture, barrier function, and homeostasis (20, 29). Our finding that undernutrition is associated with decreased proliferation and increased apoptosis is consistent with results from other published reports in animal models and humans (63, 70, 74). Similar to other rodent models of starvation or malnutrition (63, 72), mice fed the RBD developed villous blunting and decreased crypt depth, i.e., true mucosal atrophy. In contrast, human malnutrition-associated/tropical enteropathy is typically characterized by villous blunting and crypt hypertrophy (67); however, in a well-studied cohort of Zambian adults with tropical enteropathy, decreases in villous height closely paralleled decreases in crypt depth at baseline, over time, and seasonally (36). In addition, studies in Colombian patients with protein malnutrition showed that the degree of villous blunting correlated well with the severity of malnutrition, but that atrophic, normal, and hyperplastic crypts appeared to be randomly distributed among patients with varying degrees of malnutrition (19). Although regional, individual, and species differences in malnutrition-associated enteropathy are not yet fully understood, it is postulated that crypt hypertrophy may result from interactions of malnutrition, bacterial overgrowth, and repeated or prolonged enteric infections (39, 67). This view is supported by recent results from Coutinho et al. (14), in which undernourished mice had decreased villous height and crypt depth relative to nourished controls at baseline and then developed crypt hyperplasia and more severe villous blunting in response to Cryptosporidium infection. Interestingly, both undernourished and control mice developed crypt hypertrophy in response to cryptosporidiosis; however, undernourished mice developed crypt hypertrophy at a 1 log lower inclusions (14).

Important to the overall relevance of the present study to human disease, we observed that the villous height-to-crypt depth ratio (VH/CD) of undernourished, untreated controls was 4.97. In contrast, mean VH/CD between 6 and 7 were seen for Ala-Gln-treated undernourished mice and well-nourished controls. The magnitude of these differences is similar to what has been reported in jejunal biopsies from children with untreated, symptomatic, nonceliac enteropathies (VH/CD ranging from 0.85 to 1.62) and treated or asymptomatic controls (VH/CD ranging from 1.88 to 3.32) (37, 54).

This study provides the first in vivo evidence of Ala-Gln effects on both intestinal epithelial effects proliferation and apoptosis in the setting of undernutrition; however, previous studies have shown intestinal proliferative or anti-apoptotic effects of Gln or Ala-Gln in animal models of Clostridium difficile colitis (8), chemotherapy-induced mucositis (9), and gut ischemia-reperfusion injury (64). Our in vitro data show that Ala-Gln promotes the proliferation and survival of serum-starved MSIE cell line, a nontransformed, conditionally immortalized MSIE cell line. A number of studies have reported similar effects of glutamine in rat and human intestinal epithelial cell lines (18, 22, 23, 38, 52, 53) and provide evidence of glutamine regulation of specific proliferative and apoptotic signals in enterocytes, including ERK-1/2 (38, 55, 56), phosphatidylinositol-3-kinase-Akt (38), mammalian target of rapamycin (49), EGF receptor (62), heat shock proteins (21, 71), and sp3 (2), as well as signals that regulate autophagy (59). Given the significant effects we observed in our mouse model of malnutrition enteropathy, further studies are needed to determine the role of these signaling pathways in Ala-Gln-mediated intestinal homeostasis. Implications of such studies would extend beyond malnutrition to encompass other gastrointestinal diseases for which Ala-Gln supplementation has shown some benefit in metanlyses or small clinical studies. These include postsurgical infections (68), acquired immunodeficiency syndrome-associated diarrhea and wasting (7), and chemoradiation enteropathy mucositis (11).

We conclude that 1) Ala-Gln supplementation ameliorates the effects of malnutrition on intestinal mucosal architecture and barrier function, and 2) Ala-Gln reduces apoptosis and increases proliferation of mouse small intestinal epithelial cells both in vitro and in vivo. Further studies are needed to define the molecular mechanisms by which Ala-Gln mediates these cellular responses of critical importance to gut homeostasis and repair.

ACKNOWLEDGMENTS

The authors thank Celina Viana de Araujo, Antonio Ricardo Barreto, Tristan Bourdeau, Jeff Henry, and Catherine Kamiński for valuable technical assistance, and Angela Cain for assistance in preparing the manuscript.

GRANTS

This study was supported by a Pediatric Scientist Development Program award sponsored by the Eunice Shriver Kennedy National Institute of Child Health and Human Development (NICHID) (Grant award K12-HD000850 to S. R. Moore); NICHID Grant award K12-HD028827 (to S. R. Moore); Cincinnati Children’s Research Foundation support (to S. R. Moore); National Institutes of Health (NIH) Grants R01-HD053131 (to R. B. Oriá and R. L. Guerrant) and R01-DK56008 (to D. B. Polk); and, in part, by Public Health Service Grant P30 DK 078392.

DISCLAIMER

The content is solely the responsibility of the authors and does not necessarily represent the official views of NICHID, National Institute of Diabetes and Digestive and Kidney Diseases, or the NIH.

REFERENCES


