Glutamine transporter in crypts compensates for loss of villus absorption in bovine cryptosporidiosis

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Departments of 5Anatomy, Physiological Sciences, and Radiology, 1Clinical Sciences and 2Food Animal Health and Resource Management, College of Veterinary Medicine, North Carolina State University, Raleigh 27606, 3Department of Pediatrics, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599; and 5Division of Geographic Medicine, Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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Blikslager, Anthony, Elaine Hunt, Richard Guerrant, Marc Rhoads, and Robert Argenzio. Glutamine transporter in crypts compensates for loss of villus absorption in bovine cryptosporidiosis. Am J Physiol Gastrointest Liver Physiol 281: G645–G653, 2001.—Cryptosporidium parvum infection represents a significant cause of diarrhea in humans and animals. We studied the effect of luminally applied glutamine and the PG synthesis inhibitor indomethacin on NaCl absorption from infected calf ileum in Ussing chambers. Infected ileum displayed a decrease in both mucosal surface area and NaCl absorption. Indomethacin and glutamine or its stable derivative alanyl-glutamine increased the net absorption of Na+ in infected tissue in an additive manner and to a greater degree than in controls. Immunohistochemical and Western blot studies showed that in control animals neutral amino acid transport system ASC was present in villus and crypts, whereas in infected animals, ASC was strongly present only on the apical border of crypts. These results are consistent with PGs mediating the altered NaCl and water absorption in this infection. Our findings further illustrate that the combined use of a PG synthesis inhibitor and glutamine can fully stimulate Na+ and Cl− absorption despite the severe villous atrophy, an effect associated with increased expression of a Na+-dependent amino acid transporter in infected crypts.

sodium absorption; sodium/hydrogen exchanger; villous atrophy; crypt hyperplasia; prostaglandin E2

THE USE OF ORAL REHYDRATION solutions (ORS) containing glucose has become one of the most important therapeutic advances in the last century for the treatment of diarrheal disease. These ORS became well established in the treatment of cholera and other enterotoxigenic bacterial diarrheas that resulted in toxin-mediated increases in intestinal epithelial cAMP or cGMP but produced no structural damage. The effect of raising these intracellular second messengers was to inhibit an electrically neutral NaCl absorptive mechanism and to elicit anion secretion; however, the glucose-linked Na+ absorption on the small intestinal villus remained intact. This physiological principle provided the rationale for the use of oral glucose-containing electrolyte solutions, which were capable of stimulating NaCl and water absorption, thereby counteracting the second messenger-mediated events (37). Nevertheless, there are several potential difficulties with this concept in diarrheal illnesses in which there is structural damage to the intestinal mucosa. For example, the Na+-glucose cotransporter (SGLT1) has been localized to the oldest cells in the terminal villus (18). In most enteric viral diseases and those caused by invasive or effacing organisms, including Cryptosporidium, these are the first cells lost and last to be replaced in the damage-repair cycle of the villus. Furthermore, there is emerging evidence that SGLT1 may be specifically inhibited in certain viral infections (15, 21), even if these are not accompanied by significant structural damage (15).

Previous studies of cryptosporidiosis (3) or rotavirus (21, 35) in piglet models have shown that indeed, the Na+-glucose driven absorption is severely attenuated. However, in both models (4, 35) it was shown that luminally administered glutamine was capable of fully restoring Na+ transport despite the villous atrophy. Thus glutamine, a preferred fuel of the small intestine, was able to powerfully stimulate Na+ absorption in both these infections even though the absorptive epithelium on the villus was comprised exclusively of juvenile cells. In the rotavirus model, alanine was similarly as effective, suggesting that the effect of glutamine was not simply the provision of a superior metabolic substrate but might involve a regulation or distribution of amino acid transport systems that differ from the Na+-glucose transporter in infected tissue. Na+-dependent amino acid transport systems are complex and overlapping. For example, system ASC,

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an Na⁺-dependent system for neutral amino acids, is typically assigned to the basolateral membrane; however, studies of membrane vesicles from guinea pig ileum have identified the ASC system as the major Na⁺-dependent carrier for neutral amino acids in the brush border (16). In addition, a recent reexamination (31) of amino acid uptake across the mucosal membranes of intact rabbit ileum has shown that a low-affinity system for glutamate uptake, previously thought to represent system B, actually is the equivalent of system ASC. System ASC has also been shown (32) to be responsible for part of the glutamate uptake across the apical border of pig ileum. Theoretically, the presence of ASC on both apical and basolateral membranes would allow the major fuel glutamine to be taken up from either lumen or blood during digestive or interdigestive periods, respectively, whereas vectorial transport could be accomplished by Na⁺-independent systems on the basolateral membranes.

Preliminary studies with isolated cells recently showed that system ASC, which was credited with 50% of the glutamine uptake, was not only present on villus cells but also mechanistically expressed in isolated crypt cells of the guinea pig small intestine (12). Similarly, studies (29) with the rat intestinal crypt cell line IEC-17 have shown a low-affinity, Na⁺-dependent system resembling ASC to be present in these cells. Although cells in these latter studies do not display polarized membranes, it is possible that an apically located ASC transporter in the crypt of intact tissue could compensate for diminished absorption on the atrophic villus. The present study examines this hypothesis in a calf model of Cryptosporidium infection.

The calf was selected for study because it is the principal natural host for Cryptosporidium and exhibits a profuse, watery diarrhea resembling the cholera-like diarrhea in immunodeficient humans infected with Cryptosporidium.

**MATERIALS AND METHODS**

Experimental animals were 1-day-old Holstein calves obtained from a local farm and housed in isolation facilities at the College of Veterinary Medicine. All procedures were approved by the University Animal Care and Use Committee. Calves were evaluated for colostral antibody status and fed an antibiotic-free synthetic diet twice daily at a rate of 10% body wt/day (Purina kid milk replacer). Rectal swabs were taken daily from all calves and examined for the presence of Cryptosporidium. All calves infected with Cryptosporidium were positive for the organism, and control calves were always negative.

Purified *Cryptosporidium parvum* oocysts were obtained from Pleasant Hill Farm (Troy, ID). An inoculum of 10⁸ oocysts was given orally to the calves on day 7 of life. Control and infected calves were killed on day 4 postinfection, a time determined previously to be at the peak of diarrhea (unpublished observations). Calves were given a lethal overdose of intravenous pentobarbital sodium, and sections of ileum beginning 10 cm proximal to the ileocecal valve were taken for in vitro studies.

**Tissue preparation.** The section of ileum was opened along the antimesenteric border in a dissection tray and bathed in an oxygenated Ringer solution. The outer muscular layers were removed by blunt dissection, and part of the tissue was fixed in formalin for light microscopy and immunohistochemistry or snap frozen in liquid nitrogen for Western blot analysis.

**Morphometric analysis.** Formalin-fixed tissues were embedded in paraffin, cut in slices 5 μm thick, and stained with hematoxylin and eosin for light microscopy. Four to five well-oriented villi from eight infected and eight control calves were measured to determine mean villus height and diameter. Villus measurements were converted to a three-dimensional parameter using equations for a cylinder to yield villus surface area, as described previously (3).

**Gel electrophoresis and Western blotting.** Tissues were stored at −70°C before preparation for SDS-PAGE, at which time they were thawed at 4°C. Tissue portions (1 g) were added to 3 ml of chilled RIPA buffer (0.15M NaCl, 50 mM Tris, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, and 0.1% SDS), including protease inhibitors. This mixture was homogenized on ice and centrifuged at 4°C, and the supernatant was saved. Protein analysis of extract aliquots was performed (DC protein assay, Bio-Rad, Hercules, CA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 10% SDS-polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birmingham, UK) using an electroblotting minitransfer apparatus according to the manufacturer's protocol. Membranes were blocked at room temperature for 60 min in Tris-buffered saline plus 0.05% Tween 20 (TBST) and 5% dry powdered milk. Membranes were washed twice with TBST and incubated for 1 h in primary antibody (anti-ASCT-1, Chemicon, Temecula, CA). After being washed three times for 10 min each with TBST, the membranes were incubated for 45 min with horseradish peroxidase-conjugated secondary antibody. After being washed three additional times for 10 min each with TBST, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent according to the manufacturer’s instructions (Amersham, Princeton, NJ). Densitometry was performed on select blots using appropriate software.

**Immunohistochemistry.** Tissues were fixed in 10% neutral buffered formalin, routinely processed for paraffin embedding, and cut into 5-μm sections. After placement on slides, sections were deparaffinized and rehydrated. Slides were subsequently incubated in 3% H₂O₂, washed, and subjected to Pronase digestion for 10 min. Slides were washed four times in PBS between 20-min incubations with biotinylated goat anti-rabbit antibody and streptavidin-labeled peroxidase (Biogenex, San Ramon, CA). Slides were then placed in 3-amino-9-ethylcarbazole, washed in distilled water, counterstained with 0.5% methyl green for 30 s, and mounted.

**Ussing chamber studies.** Methods used in this laboratory for in vitro studies have been described in detail previously (2). Briefly, tissues stripped of the muscularis were mounted in Ussing chambers and bathed on both surfaces with 10 ml Ringer solution. Serosal glucose (10 mM) was osmotically balanced with mucosal mannitol (10 mM). In some experiments, tissues were stripped and bathed in indomethacin (1 μmol/l). In other experiments, 10 mM glutamine was added.
to the mucosal side of the tissues, and this was balanced with 10 mM mannitol added to the serosal solution. PGE₂ (10⁻⁶ M) or bumetanide (10⁻⁴ M) was added to the serosal solution in some experiments. Solutions were oxygenated and circulated with 95% O₂-5% CO₂ in water-jacketed reservoirs maintained at 39°C.

The spontaneous potential difference (PD) was measured with Ringer-agar bridges connected to matched calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes, using an automatic voltage clamp that corrected for fluid resistance. Tissues were given 30 min to equilibrate, then the isotopes²²Na and ³⁶Cl were added to the mucosal or serosal solutions. PGE₂ or bumetanide (10 M) was added to the serosal solution. PGE₂ (10⁻⁶ M) was calculated from the spontaneous PD and short-circuit current (Isc) or by clamping the tissue at ±100 μA for 5 s and recording the PD.

Statistical analysis. Data were analyzed using a paired t-test for paired treatments or a one-way ANOVA for multiple comparisons followed by a Tukey’s test to determine differences among treatments (Sigma Stat, Jandel Scientific, San Rafael, CA).

RESULTS

Cryptosporidium infection induces villous atrophy and crypt hyperplasia. As shown in Fig. 1, ileal mucosa from infected calves examined at the peak of diarrhea (postinfection day 4) displayed shortened villi and deepened crypts, which is characteristic of the entire small intestine during Cryptosporidium infection in these animals (17). Surface area calculations, based on a three-dimensional construct of the villus, showed a similarly reduced villus absorptive surface area (P < 0.05).

Cryptosporidium infection alters ion transport and Gt in calf ileum. Unidirectional and net ion fluxes, together with the Isc and Gt for control and infected calf ileum, are shown in Table 1. The infection reduced net Cl⁻ absorption and increased Isc. Unidirectional fluxes of Na⁺ and Cl⁻ were reduced in both directions, and this was paralleled by a significant reduction in Gt. Net Na⁺ absorption was reduced by one-half; however, this effect was not statistically significant.

Because control tissue demonstrated no significant basal Isc, whereas infected tissue showed a positive Isc, we next treated the serosal side of infected tissue with bumetanide (10⁻⁴ M), a specific inhibitor of the Na⁺-K⁺-2Cl⁻ uptake process on the serosal membranes. Bumetanide, at a dose shown to fully inhibit PG-stimulated Cl⁻ secretion in piglet ileum (4), reduced Isc from 12 ± 2 to 4 ± 1 μA/cm² (n = 3), illustrating that the majority of Isc in infected tissue represented Cl⁻ secretion.

Indomethacin enhances ion absorption in infected ileum. Previous studies with infected piglet ileum had shown that elevated levels of tissue PGs inhibited Na⁺ and Cl⁻ absorption and induced anion (Cl⁻ and HCO₃⁻) secretion in that tissue (2). Therefore, the PG synthesis inhibitor indomethacin was added to calf ileum to determine if endogenous PGs similarly altered the trans-

Table 1. Effect of indomethacin on Na⁺ and Cl⁻ transport in control and infected calves

<table>
<thead>
<tr>
<th></th>
<th>Na⁺</th>
<th>Cl⁻</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Jm,sc</td>
<td>Je,sc</td>
<td>Jnet</td>
</tr>
<tr>
<td>Control</td>
<td>10.6 ± 0.5*</td>
<td>9.6 ± 0.5*</td>
<td>1.0 ± 0.3*</td>
</tr>
<tr>
<td>Infected</td>
<td>6.6 ± 0.4†</td>
<td>6.2 ± 0.3‡</td>
<td>0.5 ± 0.2*</td>
</tr>
<tr>
<td>Control + Indo</td>
<td>10.8 ± 0.5*</td>
<td>10.2 ± 0.3*</td>
<td>0.7 ± 0.3*</td>
</tr>
<tr>
<td>Infected + Indo</td>
<td>8.3 ± 0.4‡</td>
<td>6.3 ± 0.4‡</td>
<td>2.0 ± 0.2‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. Fluxes and short-circuit current (Isc) are given in μeq cm⁻² h⁻¹ and tissue conductance (Gt) in mS/cm². Indomethacin (Indo)-treated tissues were stripped and bathed in 10⁻⁶ M Indo. A 1-way ANOVA was used to compare treatment means, followed by a post hoc Tukey’s test. Jm,sc, mucosal-to-serosal flux; Je,sc, serosal-to-mucosal flux; Jnet, net flux. Means with different superscripts are significantly different from each other (P < 0.05).

Fig. 1. Villus height and crypt depth of control and infected mucosa on postinfection day 4. A 1-way ANOVA yielded significant differences in both villus height and crypt depth between control and infected mucosa.

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port of these ions. The effect of indomethacin addition to control and infected ileum is shown in Table 1. In control tissue, ion fluxes or electrical parameters were unaltered by indomethacin. In contrast, indomethacin added to infected tissue increased both net Na\(^+\) and Cl\(^-\) transport and ion absorption. This effect was the result of significant increases in the \(J_{\text{m-sc}}\) of these ions; flux in the opposite direction was not significantly affected. However, indomethacin had no effect on the altered \(I_{\text{sc}}\) or \(G_t\) induced by the infection.

**Exogenous PGs inhibit ion absorption in control ileum.** Because the addition of indomethacin had no significant effect in control tissue, we added PGE\(_2\) to control tissue to determine if this maneuver would mimic the altered transport of the infection. As shown in Table 2, PG addition abolished net Na\(^+\) and Cl\(^-\) absorption. This effect was the result of significant decreases in \(J_{\text{m-sc}}\) of these ions; \(J_{\text{s-m}}\) was not significantly affected. PG addition also increased \(I_{\text{sc}}\); \(G_t\) was not significantly affected. Thus the PGs induced an impairment in Na\(^+\) and Cl\(^-\) absorption in control ileum that was qualitatively similar to the altered transport of the infection.

**Glutamine enhances net Na\(^+\) absorption in infected ileum.** Previous studies (4) with the infected piglet had shown that glutamine was more effective than glucose in stimulating Na\(^+\) absorption. Because glutamine is reported to be unstable in solutions at room temperature, we also compared the effect of alanyl-glutamine; a stable dipeptide reported to be extensively hydrolyzed by mucosal enzymes in the intestine (26). Figure 2 shows the effect of equimolar glutamine and alanyl-glutamine on net Na\(^+\) and Cl\(^-\) transport and \(I_{\text{sc}}\) in control and infected tissue. The effect of glutamine combined with indomethacin was also examined. In control tissue (Fig. 2), both glutamine and the dipeptide increased net Na\(^+\) absorption by \(\approx 1\) \(\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}\). A significant increase in \(I_{\text{sc}}\) was also observed with both compounds, which was equivalent to the net increase in Na\(^+\) absorption (1 \(\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}\)). Cl\(^-\) transport was not significantly affected. As shown before (Table 1), indomethacin alone had no effect on NaCl fluxes or \(I_{\text{sc}}\). The addition of indomethacin and glutamine together produced no further stimulation of Na\(^+\) or Cl\(^-\) absorption than glutamine alone and even appeared to reduce glutamine-stimulated Na\(^+\) absorption and significantly reduced glutamine-induced \(I_{\text{sc}}\) \((P < 0.001)\). Similar to control tissue, both glutamine and alanyl-glutamine increased net Na\(^+\) transport and \(I_{\text{sc}}\) with equal potency in the infected tissue. Indomethacin increased net Na\(^+\) transport without affecting \(I_{\text{sc}}\). Ad-

### Table 2. Effect of PGE\(_2\) on Na\(^+\) and Cl\(^-\) transport in control ileum

<table>
<thead>
<tr>
<th></th>
<th>(J_{\text{m-sc}})</th>
<th>(J_{\text{s-m}})</th>
<th>(J_{\text{net}})</th>
<th>(I_{\text{sc}})</th>
<th>(G_t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.8 ± 0.7</td>
<td>8.9 ± 0.8</td>
<td>1.0 ± 0.2</td>
<td>7.5 ± 0.8</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>+PGE2</td>
<td>8.0 ± 0.8</td>
<td>8.3 ± 0.8</td>
<td>-0.3 ± 0.2</td>
<td>4.3 ± 0.6</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>(P)</td>
<td>&lt;0.01 NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE of \(n = 10\) animals. Fluxes and \(I_{\text{sc}}\) are given in \(\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}\) and \(G_t\) in mS/cm\(^2\). A paired \(t\)-test was used to compare treatment means. PGE\(_2\) (10\(^{-6}\) M) was added to the serosal side of the tissue for the second flux period. NS, not significant.

![Fig. 2. Effect of glutamine (Gln), alanyl-Gln (Ala-Gln), indomethacin, and Gln plus indomethacin on net NaCl fluxes (J), short-circuit current \(I_{\text{sc}}\), and conductance \(G_t\) in control and infected calf ileum. A control flux period with normal Ringer (NR control) or indomethacin Ringer (IR control) was followed by a second flux period in which 10 mM Gln or Ala-Gln were added to the mucosal side of the tissues. Significant changes in net transport were accompanied by significant changes in mucosal-to-serosal fluxes, whereas changes in the opposite direction were not significantly affected (data not shown). A 1-way ANOVA was used to compare treatments followed by a post hoc Tukey's test. Data are means ± SE; \(n = \) no. of animals; NS, not significant.](http://ajpgi.physiology.org/Downloaded from).
diation of both indomethacin and glutamine further stimulated net Na⁺ absorption to a value seven times greater than the basal level and to a value now even significantly greater than the similarly treated control (P < 0.01). In further contrast to control tissue, the glutamine-induced $I_{sc}$ was unaffected by indomethacin. Neither glutamine nor alanil-glutamine by themselves significantly altered net Cl⁻ transport; however, the further addition of indomethacin induced a significant increase in net Cl⁻ absorption compared with glutamine alone. Thus these present figures indicate that the combined glutamine and indomethacin-induced increase in total net ion absorption in infected tissue was $3.5 \pm 0.09$ vs. $0.42 \pm 1.2 \mu$eq·cm⁻²·h⁻¹ in the control (P < 0.05).

PGE₂ reverses indomethacin-induced inhibition of control $I_{sc}$. The above studies showed that indomethacin paradoxically inhibited the glutamine-induced $I_{sc}$ in control tissue but had no such effect in the infected ileum. To determine if the inhibitory effect of indomethacin observed in control ileum was due to a non-specific effect of the inhibitor, tissue bathed in indomethacin was pretreated with varying concentrations of PGE₂ before addition of glutamine. As shown in Fig. 3, PGE₂ dose dependently reversed the indomethacin-induced inhibition of $I_{sc}$.

Western blot and immunohistochemistry for ASC system. In an attempt to account for the augmented stimulation of Na⁺ transport induced by glutamine in the infected atrophic tissue, we performed Western blot analyses and immunohistochemistry for the ASC transporter in control and infected tissue. The commercial antibody used is raised against a neutral amino acid transporter, ASCT-1, part of a family of structurally related glutamate transporters. As shown in Fig. 4, densitometric analysis of the Western blots showed similar transporter protein expression in control and infected tissue after equal loading of tissue protein (n = 3). As also shown in Fig. 4, indomethacin significantly reduced the expression of the ASC protein in control tissue (P < 0.05), but had no such effect in infected tissue.

As shown for control tissue in Fig. 5, D and G, the ASC system was expressed on both basal and apical borders of villus epithelia. The protein was also detected in the crypts, but with diminished intensity (Fig. 5E). Intense staining for ASC in infected tissue was seen on the apical border of the crypt epithelium (Fig. 5F). Very little staining could be seen on the infected villus epithelium, although it could be detected on some cells. Tissues stained with secondary antibody alone (negative controls) showed no evidence of stain uptake (Fig. 5, A–C).

DISCUSSION

Cryptosporidiosis is a zoonotic disease and a major cause of diarrhea in calves and humans (13). Presently, there is no effective antimicrobial treatment, and therapy is restricted to oral or intravenous rehydration. The pathophysiology of the diarrhea is complex. Detailed studies of the piglet model of cryptosporidiosis have identified an impairment in Na⁺-coupled glucose absorption, presumed due to villus atrophy (3). In addition to this structural pathogenesis, increased tissue PG levels were shown to mediate inhibition of an electrically neutral NaCl absorptive process and to elicit an electrogenic Cl⁻ and HCO₃⁻ secretory process (2). Thus net Na⁺ absorption was strongly inhibited.

Fig. 3. Effect of PGE₂ replacement on basal $I_{sc}$ and Gln-stimulated $I_{sc}$ in indomethacin-treated tissues. After tissues bathed in normal or indomethacin Ringer were mounted, PGE₂ at the concentrations shown was added to the serosal side of indomethacin-treated tissues and after a 20-min equilibration period, $I_{sc}$ was averaged for a 30-min period (period 1). Gln (10 mM) was then added to the mucosal side of the tissues, and, after a 20-min equilibration, $I_{sc}$ was averaged over the next 30 min (period 2). The Gln stimulation of $I_{sc}$ seen in normal Ringer was inhibited in indomethacin Ringer; however, addition of PGE₂ reversed the inhibition, and, at concentrations of $10^{-6}$ M, the Gln-stimulated $I_{sc}$ was fully restored (P < 0.05, n = 4).

Fig. 4. Western blot of ASCT-1 transport protein in control and infected calf ileum. A dense band in the region of 60–65 kDa was apparent in all tissue extracts. Densitometric analysis of n = 3 blots using tissues from different animals showed that the density of the ASCT-1 band in control tissue stripped and bathed in indomethacin was significantly less than in tissues bathed in Ringer (*P < 0.05).

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As shown for several species, including humans, pigs, and rabbits, the neutral NaCl absorptive process represents two apically located independent Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)\(^-\) exchange mechanisms, whose degree of coupling is dependent on the intracellular pH and whose action in the absence of substrate-coupled Na\(^+\) absorption accounts for most, if not all, of the net Na\(^+\) and Cl\(^-\) absorption in the ileum of these species (9). PGE\(_2\) and other cAMP agonists are capable of inhibiting the action of these neutral exchanges, possibly by action involving protein kinase A-mediated phosphorylation of the Na\(^+\)/H\(^+\) exchanger (27). In contrast, cAMP elevations in the crypt generally induce Cl\(^-\) secretion by phosphorylating the apical Cl\(^-\) channel; in some instances, HCO\(_3\)\(^-\) secretion is also activated, but by a poorly understood mechanism (9). In piglet ileum, both the PG-mediated anion secretory processes of Cl\(^-\) and HCO\(_3\)\(^-\) are electrogenic, and in the absence of Na\(^+\)-coupled substrate absorption, were shown to fully account for \(I_{sc}\) (4).

The source of the elevated tissue PGs in Cryptosporidium infections has not been definitively established but may be the result of infiltrating macrophages and polymorphonuclear neutrophils (3, 20), whose products have been shown to induce PG synthesis from mesenchymal cells in the lamina propria (6). Furthermore, recent studies of human intestinal epithelial cell lines showed that *C. parvum* infection directly activates PGH synthase 2 expression and PG production by these cells (23). Thus PGs could act on villus epithelium in an autocrine or paracrine manner. The present studies in the calf are consistent with a PG-mediated inhibition of neutral NaCl absorption. Thus indomethacin treatment of infected tissue restored net rates of

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**Fig. 5.** Immunohistochemical analysis of control and infected calf ileum for ASCT-1 protein. Control tissue showed stain uptake of upper (D) and lower villus and crypt (E) and on both apical and basal membranes (G). Infected ileum showed intense staining that can clearly be seen only on apical border of crypt epithelium (F). Negative controls showed no stain uptake (A–C). Scale bar for A–E = 100 µm; scale bar for G = 50 µm.
NaCl absorption to normal, whereas addition of PG to control tissue inhibited the net absorption of these ions. Interestingly, in contrast to the pig model (4), Na\(^+\) and Cl\(^-\) transport processes appear loosely coupled because indomethacin strongly increased neutral Na\(^+\) absorption, whereas its effect on net Cl\(^-\) absorption was less dramatic and not consistently significant. Furthermore, indomethacin failed to abolish the bumetanide-sensitive \(I_{\text{sc}}\) in infected calf ileum, suggesting that as-yet-unidentified, noncyclooxygenase products contribute to Cl\(^-\) secretion in this tissue.

The current studies of infected tissue also showed that luminal glutamine plus indomethacin stimulated Na\(^+\) absorption in an additive manner and emphasize the finding that despite the villous atrophy, maximal rates of NaCl absorption can be established. Similar results were obtained with glutamine in the Cryptosporidium-infected piglet (4); however, the mechanism of the glutamine-stimulated events appears to differ in the two species. In the piglet models of both rotavirus (35) and Cryptosporidium (4), the primary effect of glutamine was to strongly stimulate electroneutral NaCl absorption. For example in indomethacin-treated, Cryptosporidium-infected tissue, glutamine stimulated net Na\(^+\) absorption by 4.3 \pm 0.7 \text{ meq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}, whereas the increase in \(I_{\text{sc}}\) was only 0.8 \pm 0.1 \text{ meq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1} (4). In contrast, in the present study of the calf, the net increase in Na\(^+\) absorption induced by glutamine was numerically equal to the glutamine-stimulated \(I_{\text{sc}}\), and no significant net change in Cl\(^-\) absorption was demonstrable.

Recently, Abely et al. (1) calculated that the relative magnitude of the two Na\(^+\) absorptive processes stimulated by glutamine can vary from 0.10 to 3.5 (neutral/electrogenic) according to animal species, age, or pathological condition. For example, in rabbit ileum infected with an attaching, effacing strain of Escherichia coli (RDEC-1), glutamine stimulation of Na\(^+\) absorption was entirely electrogenic (33). However, in contrast to the present results, the magnitude of this process (33) was greatly reduced from the control and there also was loss of neutral NaCl absorption as well as the Na\(^+\)-dependent glucose absorptive processes in these adult rabbits. These results with infected rabbit ileum (33) are consistent with the location of the Na\(^+\) absorptive mechanisms on the crypt-villus axis in rabbit intestine. Recent in situ hybridization and immune complex studies (7, 14, 18) of adult rodent and rabbit intestine have shown that the messages for both SGLT1 and Na\(^+/H^+\) exchanger 3 (NHE3), the Na\(^+/H^+\) exchange isoform thought to mediate vectorial Na\(^+\) transport, are found only in villus cells with expression of the proteins in the most mature cells at the tip of the villus. These results support the paradigm of nutrient and Na\(^+\) absorptive processes residing on the most mature cells and could explain the impaired Na\(^+\) absorption in infections associated with villus cell loss and replacement with immature crypt-like cells.

Nevertheless, this hypothesis does not explain the completely intact neutral Na\(^+\) absorption unmasked by indomethacin in the Cryptosporidium-infected pig and calf and the full stimulation of Na\(^+\)-coupled glutamine absorption in the calf despite a clear loss of cells on the villus. One explanation for these differing results may be related to the enterocyte life span. For example, studies with tritiated thymidine showed that the age of an enterocyte at the time of sloughing at the villus tip is 9 days in the neonatal pig, whereas it is shortened to 3 days in the adult (28). Thus the relatively older epithelium on the neonatal villus may be capable of expressing the transporter at a more proximal site on the crypt-villus axis. Indeed, autoradiographic studies (38) have shown that enterocytes all along the crypt-villus axis take up alanine in the neonatal pig, whereas in the adult rabbit alanine uptake was confined to the villus tip. Although the present immunohistochemical studies in the control calf are consistent with this possibility, one would expect a compensatory increase in crypt cell production and a more rapid migration of crypt cells onto the villus in a repairing epithelium (39), which might offset the normally longer life span of the neonatal enterocyte.

An alternative explanation for our results is a site-specific increase in the expression of the Na\(^+\) transporters in the infection. Such region-specific upregulation has been demonstrated for NHE3 in villus epithelium of dexamethasone-treated rats (11). In these studies (11), glucocorticoids increased the levels of mRNA transcript for NHE3, which correlated with increased Na\(^+\) uptake into apical membrane vesicles (effects that were restricted to ileum and proximal colon). In studies of the streptozocin-diabetic rat, Na\(^+\)-dependent glutamine transport in brush-border membrane vesicles was augmented by a mechanism increasing the maximal rate of specific uptake with no change in the affinity constant (42). A similar adaptive upregulation of glutamine transport by brush-border vesicles was demonstrated in the short bowel syndrome in human patients treated with human growth hormone (19). Thus evidence exists for hormonal regulation of both the Na\(^+\)/H\(^+\) exchanger and Na\(^+\)-coupled glutamine transporter.

Based on the present immunohistochemical results, we propose that at least part of the effective stimulation of Na\(^+\) absorption lies in an increased expression of Na\(^+\)-dependent transporters in the crypts of the infected intestine. The detection of ASC immunostaining in the crypts of control tissue suggests that system ASC is normally expressed to some extent in the crypts. However, in contrast to control tissue, infected calf ileum showed a marked increase in the intensity of the stain on the luminal border of crypt epithelium, whereas very little signal was present on the infected villus. We are uncertain as to why there is an absence of stain on the infected villus, but it may be related to the apical membrane invagination of villus cells during parasite attachment (25). We have not seen parasites infecting crypt cells in the ileum of calves or piglets, whereas quantitative analysis of piglet ileum showed that 71\% of the villus cells were infected (4). Western blotting also showed that ASC protein was present in similar concentrations in homogenized control or in-
fected mucosa despite the loss of one-half of the villus surface area. Together, these findings strongly suggest an adaptive increase in expression of ASC protein and glutamine transport by the hyperplastic crypts of infected calf intestine. Whether a similar explanation applies to the Na\(^+\)/H\(^+\) exchanger requires further examination; however, the strong stimulation of neutral Na\(^+\) absorption by indomethacin to levels even greater than in control tissue (Table 1 and Fig. 2), suggests that electroneutral Na\(^+\) transporters are upregulated during infection even though their action is suppressed by the elevated PGs.

An interesting, but as yet unresolved finding in this study was the inhibitory effect of indomethacin on the glutamine transporter in control villus, whereas no such inhibition was seen for the glutamine transporter in the infected crypts. These inhibitory effects appeared due to the absence of basal PG production based on the PGE\(_2\) replacement studies. There may be at least two plausible explanations for this phenomenon. First, it is possible that elimination of the housekeeping PGs renders transporters on the villus more susceptible to a luminal injury by indomethacin than the better-protected transporters in the crypt (41). Alternatively, PGs may regulate trafficking or action of the ASC transporter. For example, there is evidence that PGE\(_2\) and other cAMP agonists can acutely upregulate SGLT1 expression on the villus by a mechanism involving hyperpolarization of the membrane potential. The failure of indomethacin to attenuate Na\(^+\)/glutamate cotransport in the infected calf tissue could be explained by the presence of alternative cAMP stimulants in the infected intestine, a possibility consistent with the failure of indomethacin to abolish the Cl\(^-\) secretory process. Obviously, further study is needed to distinguish among these possibilities.

In summary, the present study has shown that maximal rates of NaCl absorption can be stimulated in infected calf intestine with the combined use of a PG synthesis inhibitor and luminally administered glutamine. This maneuver allowed uninhibited operation of the neutral Na\(^+\) absorptive mechanism as well as maximal stimulation of the electrogenic process. The addition of a nonselective cyclooxygenase inhibitor to ORS may be contraindicated because of the potential deleterious effects on the mucosal barrier. The use of selective cyclooxygenase inhibitors, however, is worthy of further examination because it may be possible to preserve PG-mediated cytoprotection while eliminating their inhibitory effect on the neutral NaCl absorptive mechanisms. Similarly, potential problems with the use of glutamine exist, because glutamine is relatively unstable in solutions kept at room temperature. However, the stable dipeptide alanyl-glutamine was shown to be as effective as glutamine in stimulating Na\(^+\) absorption. Thus the dipeptide may be advantageous for use in ORS as soon as it becomes commer-

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