Astrovirus infection induces sodium malabsorption and redistributes sodium hydrogen exchanger expression

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Abstract

Astroviruses are known to be a leading cause of diarrhea in infants and the immunocompromised; however, our understanding of this endemic pathogen is limited. Histological analyses of astrovirus pathogenesis demonstrate clinical disease is not associated with changes to intestinal architecture, inflammation, or cell death. Recent studies in vitro have suggested that astroviruses induce actin rearrangement leading to loss of barrier function. The current study used the type-2 turkey astrovirus (TAvsTV-2) and turkey poult model of astrovirus disease to examine how astrovirus infection affects the ultrastructure and electrophysiology of the intestinal epithelium. These data demonstrate that infection results in changes to the epithelial ultrastructure, rearrangement of F-actin, decreased absorption of sodium, as well as redistribution of the sodium/hydrogen exchanger 3 (NHE3) from the membrane to the cytoplasm. Collectively, these data suggest astrovirus infection induces sodium malabsorption, possibly through redistribution of specific sodium transporters, which results in the development of an osmotic diarrhea.

Introduction

Astroviruses are recognized as an endemic cause of acute gastroenteritis worldwide, with serologic studies demonstrating as high as 90% of individuals in some populations have antibodies against astrovirus (Glass et al., 2001; Koopmans et al., 1998). Astrovirus disease is typically associated with children under 2 years of age, and the immunocompromised. Clinically, astrovirus induces moderate-to-severe diarrhea, abdominal pain, and vomiting; however, astrovirus diarrhea is generally less severe than that caused by rotaviruses or noroviruses and rarely requires hospitalization (Walter and Mitchell, 2003). The fact that astrovirus infection rarely requires clinical intervention makes it difficult to determine its disease burden; however, some investigators have estimated the total number of astrovirus-mediated episodes could be greater than those induced by noroviruses (Clark and McKendrick, 2004; Jakab et al., 2003).

Despite its well-recognized impact on human health, our understanding of how astroviruses cause disease is limited. This is largely due to the lack of a small animal model to study the complex interactions between the virus and the intestinal epithelium. The majority of what is known about astrovirus pathogenesis has come from studies using the turkey model (Behling-Kelly et al., 2002; Koci et al., 2003; Thouvenelle et al., 1995a,b). These experiments have demonstrated infection induces severe diarrhea in the absence of significant changes in the intestinal morphology. This includes a lack of changes in villus height, width, or surface area. Furthermore, there is no evidence of inflammation and no increase in cell death following astrovirus infection (Behling-Kelly et al., 2002; Koci et al., 2003; Thouvenelle et al., 1995a). The lack of significant histological changes following infection has also been described in biopsy samples from astrovirus-infected children (Sebire et al., 2004).

Collectively, these observations indicate astroviruses induce diarrhea through a mechanism independent of viral-mediated destruction of the epithelium, leading some to speculate astroviruses affect the physiological function of intestinal epithelial cells. Recent in vitro studies examining the effect of astrovirus infection on Caco-2 cells have supported this hypothesis and suggest the astrovirus capsid protein alone can induce actin rearrangement, resulting in increased paracellular permeability and presumably diarrhea (Moser et al., 2007).

To further understand how changes in the cellular ultrastructure are associated with clinical disease, we examined the ultrastructural and electrophysiological changes of the intestinal epithelium using the type-2 turkey astrovirus (TAvsTV-2) animal model. The results of the present study are the first to our knowledge to examine the pathophysiology of astrovirus in vivo and indicate infection leads to changes in epithelial cell ultrastructure, including actin rearrangement, in the absence of electrophysiological evidence of changes in barrier permeability. In addition, these data demonstrate infection...
induces malabsorption of Na⁺ ions associated with redistribution of the Na⁺/H⁺ exchanger, NHE3.

**Results**

**Astrovirus-induced diarrhea occurs in the absence of histological and morphological changes**

Previous studies in our laboratory and others have demonstrated astrovirus infection induces unremarkable histological changes, which do not explain the severity of diarrhea (Behling-Kelly et al., 2002; Koci et al., 2003; Sebire et al., 2004; Snodgrass et al., 1979; Thouvenelle et al., 1995a; Woode et al., 1984). The focus of the current study was to identify submicroscopic pathophysiological factors associated with astrovirus disease in vivo. To that end, tissues from the current study were first examined to confirm no overt histological changes associated with the diarrhea.

Naive animals were orally inoculated and monitored for signs of clinical disease and virus replication, as previously described (Koci et al., 2003). Individually infected animals began demonstrating clinical signs (yellow, watery, and frothy diarrhea) within 24 h of inoculation. The numbers of animals presenting with clinical signs increased daily, with 100% of inoculated animals exhibiting diarrhea by 3 dpi (data not shown). The severity of clinical signs persisted for approximately 4 days followed by a recovery period that lasted until the end of the experiment. The kinetics of the clinical disease and correlation with the level of virus in intestinal samples and feces, as detected by real-time RT–PCR (data not shown), was similar to previous reports (Koci et al., 2003).

At necropsy, the intestines of infected animals were found to be distended and filled with frothy fluid, consistent with diarrheal disease. Hematoxylin and eosin staining and light microscopic examination of the duodenum, jejunum, ileum, and cecum demonstrated no notable changes between infected and control animals, including a lack of villus blunting, crypt hyperplasia, or epithelial sloughing (Fig. 1). In addition, morphometric assessment of the villous height, villous width, villous surface area, crypt depth, villous–crypt depth ratio, and number of goblet cells per villi revealed no significant differences between control and TAstV-2–infected samples (data not shown). These observations were consistent with previous reports and demonstrate that TAstV-2–induced diarrhea occurs without light microscopic mucosal changes (Behling-Kelly et al., 2002; Koci et al., 2003).

**TAstV-2 infection results in ultrastructural changes and actin rearrangement in intestinal epithelial cells**

To determine if astrovirus–induced clinical disease was associated with submicroscopic changes in the intestinal epithelium, sections of the jejunum were collected from control and infected animals at 4 dpi (12–24 h after onset of severe clinical signs in 100% of inoculated animals) and processed for analysis by TEM. TEM analysis demonstrated that epithelium from control tissues had normal appearance with clearly demarcated tight junctions, whereas epithelium from TAstV-2–infected tissues had evidence of electron dense aggregates within the apical region of the enterocytes (Fig. 2). These electron-dense aggregates were more prominent in the epithelial cells at the tips of villi and were less frequently noted toward the intestinal crypts within the basal region of the mucosa (Fig. 2). Electron-dense aggregates appeared to be specifically associated with tight junctions, and there was no evidence of paracellular dilatation in these regions (Fig. 2). The electron-dense aggregates appeared to be clustered around filaments extending from the microvilli, suggesting viral-induced alteration of the cytoskeleton.

To investigate if electron-dense aggregates were associated with actin rearrangement, tissue sections were assayed for changes in F-actin using fluorescent–labeled phalloidin. F-actin in control tissues appeared as a sharp fluorescence demarcating individual enterocytes, with some evidence of intracellular stress fibers (Fig. 3A). In contrast, the TAstV-2–infected jejunum revealed a poorly defined and thickened layer of F-actin fluorescence along the apical border of enterocytes with focal areas of aggregations (Fig. 3A).

Analysis of tissues stained for both F-actin and TAstVnsp suggested a correlation between areas of the intestine positive for virus replication and areas with changes in F-actin (Fig. 3A). To better assess this relationship, tissues were analyzed to determine the percent of tissues where both events could be observed in the same 25 μm grid. This analysis demonstrated that TAstVnsp was found in approximately 80% of the tissue from infected animals, with approximately two-thirds of these infected regions to have moderate to severe changes in actin (Fig. 3B). More specifically, these fluorescent images were examined for colocalization of TAstV2nsp and F-actin. The results of these analyses demonstrated significant colocalization (overlap coefficient = 0.98, P < 0.05) between F-actin and TAstV2nsp fluorescence.

To further characterize the actin rearrangement observed in the TAstV-2–infected small intestinal epithelium, immunoblotting was used to study total actin content and the proportion of G- and F-actin in TAstV-2–infected intestinal mucosa. The total actin content in mucosal homogenates, as assessed by pan-actin antibody staining, was not different in TAstV-2–infected tissue as compared to control tissues (data not shown). Moreover, the ratio of G- and F-actin was also found to be unchanged after TAstV-2 infection (data not shown). Thus, the rearrangement of apical F-actin after TAstV-2 was found to be limited to morphological alterations without changes in actin content.

**Ultrastructural changes are not associated with changes in mannitol permeability**

To further characterize the pathophysiological significance of the ultrastructural changes, sections of jejunum were assayed for changes in paracellular permeability by assaying for differences in mucosal-to-serosal fluxes (Jms) of [3H]-mannitol across the jejunum of TAstV-2–infected and control animals. There was no significant difference between Jms mannitol in control versus infected tissues (0.61 ± 0.06 vs. 0.53 ± 0.04 μmol/cm²/h, respectively, P > 0.05); however,
conductance ($G$) was found to be increased in TAstV-2-infected animals as compared to controls (Table 1). This suggests that although macromolecular permeability was not altered, as measured by flux of mannitol, there were changes in permeability at the ionic level, as measured by $G$.

**TAstV-2-associated change in ion transport**

To further characterize the effect TAstV-2 infection had on the electrophysiological properties of the gut, jejunal sections were assayed for changes in ion transport as reflected by short circuit current ($I_{sc}$) and defined by unidirectional fluxes of Na$^+$ and Cl$^-$ (Argenzio and Liacos, 1990; Blikslager et al., 1999; Li et al., 2004). The $I_{sc}$ values of jejunum from infected animals did not indicate the presence of a typical electrogenic secretory response in which the principal ion secreted is anionic (Table 1). This was confirmed by analysis of the Na$^+$ and Cl$^-$ transport. No significant differences in mucosal-to-serosal, serosal-to-mucosal, or net flux of Cl$^-$ (Table 1) were observed. In addition, no significant difference in serosal-to-mucosal flux of Na$^+$ was detected between the jejunum of control and infected animals (Table 1); however, mucosal-to-serosal Na$^+$ flux was significantly reduced in TAstV-2-infected animals. Accordingly, net Na$^+$ absorption was significantly reduced in TAstV-2-infected tissues (Table 1). These findings indicated that infection was associated with malabsorption of Na$^+$. Although Na$^+$ absorption is typically electroneutral (no change in $I_{sc}$), it is possible that Na$^+$ absorption was reduced to the extent that it contributed to changes in measurements of $I_{sc}$ (Martinez-Augustin et al., 2009).

**Dysregulation of sodium hydrogen exchangers in TAstV-2-infected animals**

Based on the observations that TAstV-2 infection impaired absorption of Na$^+$, we assayed for changes in expression of sodium hydrogen exchangers (NHE). NHE2 and NHE3 have been described as the principal intestinal brush border NHE isoforms contributing to electroneutral Na$^+$ absorption in the avian small intestine (Donowitz et al., 1998). To assay for their relative level of expression, Western blot analyses of jejunal mucosal scrapings were performed. The level of NHE2 in whole tissue lysates was increased in the TAstV-2-infected animals as compared to controls (Fig. 4D). Alternatively, there was no significant difference in the level of NHE3 protein (Fig. 4A). These changes were difficult to interpret given our electrical findings, leading us to study NHE expression within cell fractions. Because NHE2 and NHE3 reside in the plasma membrane, we assayed for a difference in their expression in detergent-soluble and detergent-insoluble fractions. Detergent-soluble fractions would be expected to contain proteins contained within the cytoplasm, whereas insoluble fractions would include the apical membrane in addition to other hydrophobic proteins. The results of these assays demonstrated a significant shift in the location of NHE3 within the cell following infection. The TAstV-2-infected tissues had approximately half the amount of NHE3 associated with the insoluble fraction as compared to controls.
the controls (Figs. 4B and C). No significant difference was observed in the cellular localization of NHE2 (Figs. 4E and F).

Discussion

Understanding how viruses induce diarrhea and the specific changes to the host’s intestinal physiology is essential to the development of more effective therapies. This is particularly important with a pathogen like astrovirus that has the heaviest disease burden in the immunologically immature and immunocompromised host. Previous studies of astrovirus pathogenesis in humans and animals suggest infection causes mild changes to the villus architecture, no change in absorptive surface area, and limited evidence of inflammation (Behling-Kelly et al., 2002; Koci et al., 2003; Sebire et al., 2004; Snodgrass et al., 1979; Thouvenelle et al., 1995a; Woode et al., 1984). Studies by Thouvenelle et al. (1995b) suggested that astrovirus infection induces changes in expression of constituent proteins of the brush border resulting in malabsorptive diarrhea, while more recent

### Table 1

Values are mean ± SE. Jejunal tissue was stripped of seromuscular layer (A) or not (B) before mounting on Ussing chambers. Na⁺ and Cl⁻ fluxes (J) and short-circuit current (Isc) are given in μeq/cm²/h, and tissue conductance (Gt) is given in mS/cm². Jm→s, mucosal-to-serosal flux; Js→m, serosal-to-mucosal flux; Jnet, net flux. n = 6 for each experiment.

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<td></td>
<td>Jm→s</td>
<td>Js→m</td>
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<td>Control</td>
<td>4.36 ± 0.31</td>
<td>3.58 ± 0.38</td>
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<tr>
<td>TAstV-2</td>
<td>2.03 ± 0.36 *</td>
<td>4.13 ± 0.42</td>
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<td>Jm→s</td>
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<tr>
<td>Control</td>
<td>4.36 ± 0.48</td>
<td>3.80 ± 0.29</td>
</tr>
<tr>
<td>TAstV-2</td>
<td>2.91 ± 0.27 *</td>
<td>4.04 ± 0.61</td>
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* P < 0.05 infected vs. control tissues (t-test).
in vitro studies by Moser et al. (2007) indicate astroviruses are capable of increasing paracellular permeability. The present study was designed to identify astrovirus-mediated changes to the ultrastructure and electrophysiology of the small intestine, which could help explain the mechanisms underlying clinical disease.

The present study focused on the jejunal region of the small intestine. The avian jejunum has been described to have more total surface area and is consequently a primary site of nutrient absorption as compared to other regions of the small intestine (Grubb and Bentley, 1990; Sklan et al., 2003). In addition, previous studies have suggested astrovirus-induced changes to brush border enzyme activity may be greatest in the jejunum (Thouvenelle et al., 1995b). Therefore, we used the TAstV-2 and naive turkey poult model to investigate astrovirus disease, including evaluation of electrophysiological parameters, mucosal-to-serosal mannitol fluxes, and unidirectional ion fluxes across the mucosa of the small intestine following the onset of severe clinical signs and virus replication. The present studies indicated that TAstV-2 infection induces malabsorption of Na⁺. This malabsorption was accompanied by a decrease in expression of NHE3 in the detergent-insoluble fraction and changes to the actin cytoskeleton associated with the epithelial brush border.

**Na⁺ malabsorption**

Fluid loss into the intestinal lumen due to infectious diarrheal disease is primarily thought to involve electrogenic chloride secretion, while malabsorptive diarrheal disease typically refers to a reduction in absorptive surface area with which to absorb crucial electrolytes. The present study demonstrated neither appears to be involved with astrovirus-induced disease. These results demonstrated a malabsorption of Na⁺ following infection. Reduced Na⁺ absorption has been associated with infectious diarrhea (Halaihel et al., 2000; Shepherd et al., 1979), inflammatory bowel disease (Hawker et al., 1980), and TNF-α-induced experimental models of diarrhea (Clayburgh et al., 2006). Impaired Na⁺ absorption leads to a reduced gradient for water absorption due to accumulation of Na⁺ in the lumen, causing malabsorptive diarrhea. What is particularly noteworthy in the present study is that astrovirus-induced diarrhea appears to be attributable to the reduction in membrane expression of a Na⁺ transporter associated with disruption of the apical membrane, which, to our knowledge, has not been previously identified as a mechanism for viral enteritis.

It should also be noted that Na⁺ absorption, however, is typically electroneutral (no change in \( I_{sc} \)), which was not the case here. Defects in the transport of ions other than Na⁺ may also exist as the net Na⁺ and Cl⁻ flux values did not fully explain the magnitude of change in \( I_{sc} \) in this study (Table 1), and thus, the residual ion flux (not shown) could represent defects in other ion transport systems possibly as a result of the cell switching from electroneutral Na⁺ absorption to electrogenic to compensate for the putative reduction in NHE3 activity (Flagella et al., 1999; Lucas, 2008). Additional studies are required to study other cations (K⁺) or anions (HCO₃⁻) that contribute...
to changes in \( I_C \) and identify other ions whose transport across the intestinal barrier may also be disrupted.

**Altered NHE expression**

NHE2 and 3 belong to the Slc9a gene family which encodes nine NHE isoforms. Each isoform has been reported to be expressed differentially throughout the intestine and within the cell (Kiela et al., 2006). NHE2 and 3 are thought to function almost exclusively in the apical membrane. They form the principal mechanism of enterocyte Na\(^{+}\) absorption and therefore form the basis of intestinal water absorption (Zachos et al., 2005). NHE2 and 3 differ greatly as far as how their expression and activity are regulated within the cell. The NHE2 protein has been reported to be regulated transcriptionally (Cavet et al., 2001), while NHE3 is regulated posttranslational through endosomal recycling (Akhter et al., 2002; Kurashima et al., 1998). Functionally, NHE3 is thought to be responsible for the majority of Na\(^{+}\) absorption in mammals (Ledoussel et al., 2001; Wormmeester et al., 1998), while in the normal chicken ileum and colon, NHE2 and NHE3 have been described to contribute equally to Na\(^{+}\)/H\(^{+}\) exchange (Donowitz et al., 1998). Expression and activity of these proteins, however, have not been well characterized in the jejunum and have not been studied in other avian species.

In the present study, we found that although total expression of NHE3 was unchanged, its expression was increased in the detergent-soluble fraction and decreased in the detergent-insoluble fraction of TAstV-2-infected intestine. The decrease in detergent-insoluble NHE3 in astrovirus-infected mucosa suggested a reduction in membrane expression of this protein. A decrease in NHE2 in the apical membrane would be expected to lead to a decrease in Na\(^{+}\) absorption. In contrast, the total expression of NHE2 was increased in TAstV-2-infected jejunum. Although the reasons for these findings are not clear, increased expression of NHE2 in TAstV-2-infected jejunum could be a compensatory response for loss of NHE3 activity (Bachmann et al., 2004).

Previous studies to investigate the role of NHEs and Na\(^{+}\) absorption during enteropathogenic *Escherichia coli* (EPEC) infections have demonstrated a differential regulation of NHE2 and NHE3 (Hecht et al., 2004). The mechanisms involved in the differential regulation of NHE2 and 3 are not understood; however, they are likely related to differences in how the proteins are regulated and differences in their cytoskeletal associations.

Currently, it is unclear if the changes in NHE3 expression following astrovirus infection are the result of specific targeting by viral proteins or a function of astrovirus-mediated changes to actin and/or interactions with other host proteins involved in linking NHE3 to the brush border. Initial studies to assay for changes in other proteins (NHERF, ezrin, ERK, etc.) were unsuccessful due to a lack of cross-reactivity with turkey tissues. Efforts are presently underway to develop model-specific reagents to further investigate the mechanisms involved in NHE3 inhibition.

**Actin rearrangement and the epithelial barrier**

The complexity of cytoskeletal–plasma membrane interactions is important for stabilization, function, and trafficking of transport proteins residing within the plasma membrane (Khurana, 2000). Apical F-actin forms a continuous ring around the epithelial cell that is anatomically linked to apical tight junctions and dynamically regulates tight junction permeability. For example, contraction of actin fibers opens the tight junctions and results in increased paracellular permeability (Madara et al., 1987; Turner et al., 1997).

The results of the experiments in the current study suggest astrovirus infection-induced changes to the epithelial cytoskeleton in vivo. These changes in F-actin appeared at the apical surface as observed first as electron dense areas under TEM (Fig. 2) and then subsequently by fluorescence (Fig. 3). Moreover, the changes in F-actin were also observed to be associated with astrovirus replication, as detected by the presence of astrovirus nonstructural protein (Fig. 3). Colocalization of the F-actin and TAstV2nsp suggests that during astrovirus replication, nonstructural proteins localize to the apical region of the infected cell, potentially interacting with host proteins and affecting actin polymerization. The results of this study, however, do not address what role other astrovirus proteins may play in the observed changes to F-actin. Previous studies have demonstrated astrovirus capsid protein alone is sufficient to induce changes in actin rearrangement in vitro (Moser et al., 2007). Additional studies are necessary to better understand what effect specific astrovirus proteins and the different stages of the virus life cycle have on the enterocyte cytoskeleton.

The changes in epithelial actin were also associated with an increase in electrical conductance (Table 1), suggesting subtle changes in permeability. However, there was no evidence of increased paracellular permeability as determined by mannitol fluxes or observation of changes in paracellular spaces as observed by TEM (Fig. 2). In addition, no changes in cellular localization of the tight junction protein ZO-1 were detected (data not shown). Collectively, these results suggest that infection was capable of inducing changes to the epithelial cytoskeleton, which, in turn, altered Na\(^{+}\) transport and electrolyte permeability without inducing overt evidence of changes in barrier function.

These findings differ in some respects with those reported by Moser et al. (2007) who reported that infection of Caco-2 cells with human astrovirus type-1 induced a loss of barrier function. The reason(s) for these differences is currently unclear; however, it could be a function of differences in the response of in vivo jejunal epithelial cells as compared to transformed colon cells (Caco-2 cells). Alternatively, the differences could be due to differences in the stage of virus infection at the time of analysis. The changes described by Moser et al. occur within 36 h of infection in vitro, whereas in the current study, intestinal epithelium was not sampled until after the onset of severe clinical signs (4 dpi). Additional studies are needed to examine the kinetics of astrovirus-mediated changes in intestinal electrophysiology in the jejunum as well as in other regions of the small intestine to better understand their association with and contribution to clinical disease.

**Conclusions**

These studies are the first, to our knowledge, to investigate the electrophysiological changes associated with astrovirus infection ex vivo. These results suggest astrovirus infection induced ultrastructural changes to the intestinal epithelium and rearrangement in F-actin. We hypothesize that these changes in actin disrupt the normal expression of transporter proteins in the apical membrane, specifically NHE3, and lead to malabsorption of Na\(^{+}\), resulting in the failure to fully absorb water and ultimately clinical diarrhea. Future studies will investigate the specific interactions between astrovirus proteins, NHE3, and actin and their association with the severity of clinical disease, as well as the disruption of absorption of other ions and solutes.

**Materials and methods**

**Animals and viral Infection**

One-day-old unvaccinated turkey poultys were obtained from a commercial hatchery. Animals were randomly divided into 2 groups and placed in separate 934-1-WP isolators (L. H. Leathers, Inc, Athens, GA) with free access to feed and water. After an acclimation period of 3 days, one group was orally inoculated with \( \sim 10^7 \) genomic units of type-2 turkey astrovirus (TAstV-2/NC/99, TAstV-2) in 100μl of phosphate-buffered saline (PBS) and one sham inoculated group.
from 3 samples and the ImageJ program (http://rsb.info.nih.gov/ij/). Samples of jejunal mucosa were collected at 4 days post infection (dpi) from 5 Con and 5 TAstV-2-infected animals for electrophysiology and unidirectional Na⁺ and Cl⁻ flux studies on Ussing chambers, electron microscopy, and Western blot analysis. Jejunal samples were also collected at 5 dpi for measuring paracellular mucosal-to-serosal ³H-mannitol fluxes on Ussing chambers. These time points correspond with the onset of severe clinical signs.

All animal procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Electron microscopy

Jejunal tissues were fixed in McDowell’s and Trump’s 4F:1G fixative and processed for transmission electron microscopy using standard techniques (Dykstra, 1993). In brief, after 2 rinses in 0.1 M sodium phosphate buffer (pH 7.2), samples were placed in sodium phosphate buffer + 1% osmium tetroxide for 1 h at room temperature. Samples were rinsed 2 times in distilled water and dehydrated in an ethanolic series culminating in two changes of 100% acetone. Tissues were then placed in a mixture of Spurr’s resin and acetone for 30 min, followed by 2 h in 100% resin with 2 changes. Finally, samples were placed in fresh 100% resin in molds and polymerized at 70 °C for 8 h to 3 days. Semithin (0.25–0.5 µm) sections were cut with glass knives and stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin (70–90 nm) sections were cut with a diamond knife, stained with methanolic uranyl acetate followed by lead citrate and examined with a transmission electron microscope (Phillips/FEICO Model 208s).

Immunofluorescence

Jejunal tissues were embedded in OCT media (Tissue Tek, Sakura Finetek, Torrance CA), frozen, sectioned at 5 µm, and stored at −70 °C until use. The sections were thawed, fixed in cold acetone, and incubated with a rabbit polyclonal antibody (anti-TAstV2nsp) generated to a peptide sequence derived from the TAstV-2 nonstructural protein (CAMEDRGFQYKKSR; GenScript Corporation, Piscataway, NJ). After washings in PBS, the sections were incubated in a mixture of goat antirabbit mouse–Alexa Fluor 488, phalloidin conjugated with Alexa Fluor 546, and TO-PRO-3 (nucleic acid stain; 30 µg/ml leupeptin, and 0.7 g/ml pepstatin A). This mixture was diluted in 10 µl of PBS as previously described (Koci et al., 2003). After a 15 min equilibration period, standards were taken from the mucosal side of each chamber. A 60 min flux period was established by taking 0.5 µl samples from the serosal compartment opposite to the mucosal compartment to which [³H]-mannitol was added. The presence of [³H]-mannitol was established by measuring β-emission in a liquid scintillation counter (1219 Rack Beta, LKB Wallac, Perkin Elmer, Waltham, MA).

To assess transmucosal Na⁺ and Cl⁻ fluxes, 22Na⁺ or 36Cl⁻ were added to the mucosal or serosal solutions of tissues paired according to their conductance (conductance within 25% of each other). After a 15 min equilibration period, standards were collected and 3 successive 30 min flux periods were performed by taking samples from the bathing reservoirs opposite the side of isotope addition. Data presented are from the second flux period. Samples were placed sequentially in β- and γ-liquid scintillation counters. The contribution of 22Na β-counts to 36Cl β-counts was subtracted, and the unidirectional fluxes were calculated as previously described (Argenzio and Armstrong, 1993).

Western blotting

Jejunal mucosal scrapings from control and infected animals were snap-frozen and stored at −70 °C. Tissue aliquots were thawed at 4 °C and added to chilled lysis buffer that included protease inhibitors (0.5 mM Pefabloc, 0.1 mM 4-nitrophenyl phosphate, 0.04 mM glycerophosphate, 0.1 mM Na₂VO₃, 40 µg/ml bestatin, 2 µg/ml aprotinin, 0.54 µg/ml leupeptin, and 0.7 µg/ml pepstatin A). This mixture was homogenized on ice, centrifuged (800 × g for 10 min at 4 °C) to remove debris and clarified by centrifugation (2000 × g for 10 min at 4 °C), and the supernatant was collected. Protein concentrations were determined (BCA Protein Assay; Pierce, Rockford, IL), and samples were normalized based on total protein concentration.

To assay for differences in membrane or cytosolic cellular localization of protein, jejunal mucosa scrapings were first homogenized in buffer (20 mM Tris, 5 mM MgCl₂, 0.3 mM EGTA, 210 µg/ml sodium fluoride, 18.5 µg/ml sodium orthovanadate, 30 mM sodium pyrophosphate, and Complete Mini Protease inhibitor cocktail tablet (Pierce)) and centrifuged to remove debris (800 × g for 5 min at 4 °C). Supernatants were then incubated for 30 min at 4 °C with lysis buffer containing 0.5% Triton X-100 and centrifuged (12,000 × g for 30 min at 4 °C), and the supernatant containing detergent-soluble proteins was collected (predominantly cytosolic proteins). The pellet was resuspended in lysis buffer containing 0.5% SDS and processed as above to obtain detergent-insoluble proteins (predominantly membrane
proteins. Before SDS–PAGE analysis, samples were processed through an SDS–PAGE sample preparation kit to remove excessive detergent. Protein concentrations were determined (BCA Protein Assay; Pierce), and samples were normalized based on total protein.

Samples for PAGE analysis were mixed with 4× XT sample buffer (Bio-Rad, Hercules, CA) and boiled for 4 min. Lysates were loaded on a and samples were normalized based on total protein.


