INFECTION AND IMMUNITY, Nov. 2006, p. 6075–6084 Vol. 74, No. 11
0019-9567/06/$08.00+0 doi:10.1128/IAI.00721-06

Evidence that Tight Junctions Are Disrupted Due to Intimate Bacterial Contact and Not Inflammation during Attaching and Effacing Pathogen Infection In Vivo▽

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Received 4 May 2006/Returned for modification 10 August 2006/Accepted 18 August 2006

It is widely accepted that tight junctions are altered during infections by attaching and effacing (A/E) pathogens. These disruptions have been demonstrated both in vitro and more recently in vivo. For in vivo experiments, the murine model of A/E infection with *Citrobacter rodentium* is the animal model of choice. In addition to effects on tight junctions, these bacteria also colonize the colon at high levels, efface colonocyte microvilli, and cause hyperplasia and inflammation. Although we have recently demonstrated that tight junctions are disrupted by *C. rodentium*, the issue of direct effects of bacteria on epithelial cell junctions versus the indirect effects of inflammation still remains to be clarified. Here, we demonstrate that during the *C. rodentium* infections, inflammation plays no discernible role in the alteration of tight junctions. The distribution of the tight junction proteins, claudin-1, -3, and -5, are unaffected in inflamed colon, and junctions appear morphologically unaltered when viewed by electron microscopy. Additionally, tracer molecules are not capable of penetrating the inflamed colonic epithelium of infected mice that have cleared the bacteria. Finally, infected colonocytes from mice exposed to *C. rodentium* for 14 days, which have high levels of bacterial attachment to colonocytes as well as inflammation, have characteristic, altered claudin localization whereas cells adjacent to infected colonocytes retain their normal claudin distribution. We conclude that inflammation plays no discernible role in tight junction alteration during A/E pathogenesis and that tight junction disruption in vivo appears dependent only on the direct intimate attachment of the pathogenic bacteria to the cells.

Humans and animals infected with the attaching and effacing (A/E) pathogens enterohemorrhagic *Escherichia coli*, enteropathogenic *E. coli* (EPEC), and *Citrobacter rodentium* suffer from serious diarrhea, or diarrhea-like conditions, that can result in death. These pathogens attach to the surface epithelial cells of the intestine and inject effector proteins through a type III secretion system (TTSS) into host cells (18, 32). These pathogenic proteins commandeer various host cell functions and structures that contribute to the disease process. Some of the host structures targeted by A/E pathogens include the host cell's microvilli (7, 24), mitochondria (19, 27, 31), and tight junctions (3, 6, 23, 26).

In intestinal epithelial cells, tight junctions are the most luminal cell-cell junctions of the apical junction complex (10). These junctions have two functions. One is to act as permeability barriers, separating the luminal environment from the adluminal region of the epithelium; the other is to segregate apically located membrane proteins from those at the basolateral membrane. Tight junctions are often viewed as membrane fusions by transmission electron microscopy because the membranes of adjacent cells are held in extremely close opposition by the transmembrane protein occludin and members of the claudin family that form the junctions (11, 13, 45). Both of these protein groups contain four transmembrane-spanning regions but do not share any sequence homology (reviewed in reference 15). They attach intracellularly to the adaptor molecules zonula occludens 1 (ZO-1), ZO-2, and ZO-3. ZO-1 links the transmembrane proteins to the actin cytoskeleton (9, 45). Work on these two groups of proteins has yielded striking differences as to their importance in maintaining the barrier function of the tight junctions. Studies using occludin null mice have demonstrated that occludin is dispensable with regard to tight junction barrier function (36, 37). These mice, devoid of occludin, have tight junctions that function normally (36). Conversely, mice deficient in all claudins targeted thus far have catastrophic tight junction deficiencies (12, 16, 30). In the colon, claudin-1 (51), -2, -3, -4, and -5 (35) are present in colonocytes.

Until recently, most research on tight junction disruption induced by A/E pathogens has focused on EPEC infections in cell culture (in vitro) (6, 23, 43, 46, 48, 50). Generally it is accepted that in vitro, EPEC causes a redistribution of the tight junction proteins occludin, ZO-1, and claudin-1 as well as a decrease in transepithelial resistance (TER) (26). These alterations are thought to be due to the effector proteins EspF and Map (mitochondria-associated protein) (6, 23, 48). We previously have used a naturally occurring A/E murine infection model to test whether tight junctions are disrupted in vivo (17). This model uses the A/E pathogen *C. rodentium* to infect mice. This pathogen colonizes the colon, collapses microvilli, and produces a diarrhea-like phenotype in infected mice (22). We demonstrated that *C. rodentium* disrupts tight junctions in

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▽ Published ahead of print on 5 September 2006.

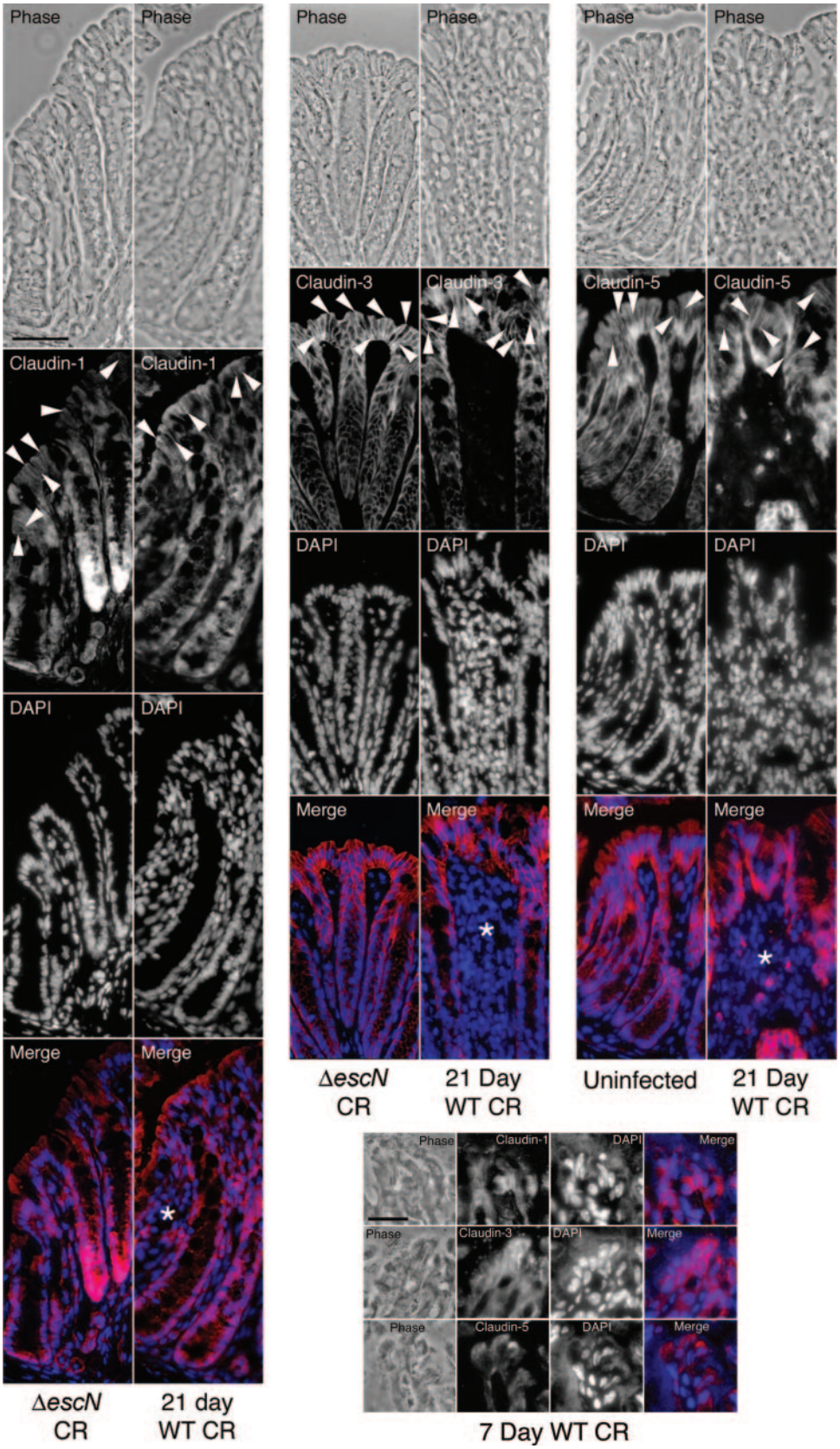


FIG. 1. Claudin-1, -3, and -5 and DAPI (DNA) localization on 21-day wild-type *C. rodentium*-infected mouse sections paired with 21-day Δ*escN* *C. rodentium*-infected tissue or uninfected tissue. Claudin staining of 7-day wild-type *C. rodentium*-infected tissue is also presented. Arrowheads indicate areas of staining at the lateral boundaries of colonocytes. The asterisks in the merged images indicate regions with high levels of cellular infiltrate in the lamina propria of 21-day wild-type *C. rodentium*-infected inflamed epithelium. WT, wild type; CR. *C. rodentium*. Scale bars, 50 μm (21-day micrographs) and 25 μm (7-day micrographs).

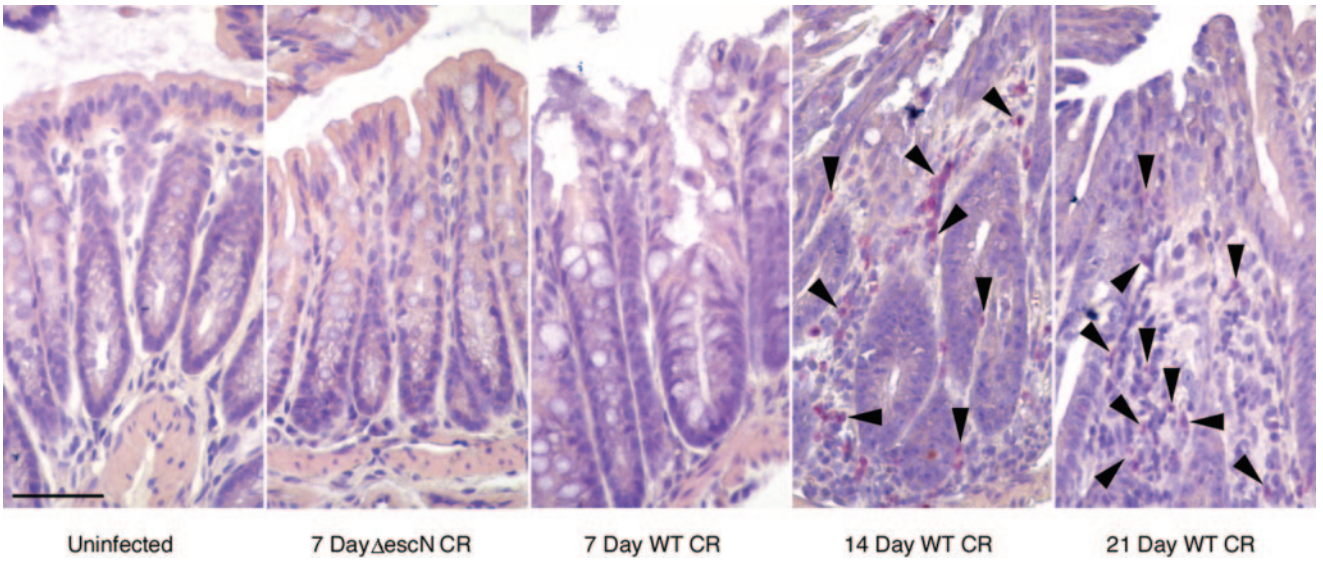


FIG. 2. Neutrophil-labeled micrographs counterstained with hematoxylin. Neutrophil labeling on uninfected, 7-day Δ*escN C. rodentium* (Δ*escN* CR), or 7-day, 14-day or 21-day wild-type *C. rodentium* (WT CR) infections. Arrowheads indicate some of the neutrophils (pink staining). Increased numbers of neutrophils are present in the lamina propria of 14- and 21-day wild-type *C. rodentium*-infected colon sections compared to the uninfected, 7-day Δ*escN C. rodentium*-infected or 7-day wild-type *C. rodentium*-infected tissue. Scale bar, 50 μm.

mice and that this disruption is dependent on the bacterial type III effector protein EspF but not Map (17).

Although the evidence is controversial, it has been hypothesized that inflammatory effects participate in tight junction alteration during infection (28). Studies have shown that the addition of tumor necrosis factor-α to cultured colonic cells (HT-29/B6) decreases tight junction strands (14) and that the addition of both tumor necrosis factor-α and gamma interferon reduces the expression of claudin-2 but not the expression of claudin-3 and -4 in T84 cells (34). However, the addition of interleukin-13 increases the expression of claudin-2, while the levels of claudin-3 and -4 are unchanged in T84 cells (34). This result occurs with an increase in dextran permeability and a decrease in TER (34). Western blots of claudin-1, -2, -3, and -5 from mice deficient in interleukin-2, which have colonic inflammation, show a dramatic increase in protein levels in membrane preparations from the colon (1). In humans with inflammatory bowel disease a report by Gassler and coworkers (13a) suggests that ZO-1, occludin, claudin-1, and claudin-2 are all reduced. These results are contrary to those in a recent report by Prasad and coworkers (34) in which they demonstrate that claudin-2 expression increases and claudin-3 and -4 expression decreases in patients with Crohn's disease and ulcerative colitis/inflammatory bowel disease.

There is some suggestion that EPEC causes the release of inflammatory mediators in vitro (in cell culture) (4, 38, 41). To date, only one study has attempted to link inflammatory effects with tight junction alteration caused by A/E pathogen infection (39). In that study EPEC-infected cells were pretreated with drugs that inhibit mitogen-activated protein (MAP) or extracellular signal-regulated kinase (ERK) kinase (39). Although these inhibitors have numerous effects on cells in general, they apparently had no effect on the decrease in TER during EPEC infection in vitro (39).

To determine what role A/E pathogen-induced inflammation has on tight junctions in vivo, we infected C57BL/6 mice with *C. rodentium* and then evaluated the status of colonic tight junctions at a time point (21 days) when bacteria have been cleared from the colon but inflammation persists. We confirmed that, at this time point, bacteria were not attached to colonocytes and that inflammatory cell infiltrate was evident in the lamina propria. Immunolocalization of claudin-1, -3, and -5 on infected tissue sections labeled the lateral boundaries of colonocytes and appeared unaltered compared to uninfected or Δ*escN C. rodentium*-infected controls. Electron microscopy validated these findings by demonstrating morphologically intact tight junctions. Functional tracer experiments showed that the tight junctions remained intact as the chemical tracers were retained in the lumen of the colon without any penetration into the mucosa. Additionally, we demonstrated that in tissue in which both bacteria are attached and inflammation persists, tight junctions are altered only where bacteria are intimately attached. These findings provide crucial in vivo evidence that the inflammatory response caused by A/E pathogens does not play a significant role in tight junction alteration; instead, it is direct bacterial contact that mediates junctional disruption.

MATERIALS AND METHODS

Chemicals and reagents. Chemicals and reagents used in this study were obtained from Sigma-Aldrich Canada (Ontario, Canada). Paraformaldehyde was acquired from Canmeco (Quebec, Canada), and NaCl was from Fisher Scientific (British Columbia, Canada). Secondary antibodies conjugated to horseradish peroxidase and control immunoglobulins (immunoglobulin G [IgG]) were purchased from Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania). All secondary antibodies conjugated to Alexa fluorochromes were purchased from Molecular Probes (Oregon), as was the Alexa 488-conjugated streptavidin.

Animals, bacterial strains, and mouse infections. Four- to six-week-old female C57BL/6 mice were acquired from the Jackson Laboratory and Charles River Laboratories. Upon arrival at the University of British Columbia animal care center, animals were left undisturbed for at least 4 days prior to oral gavage infections with 4×10^8 to 5×10^8 Δ*escN* or wild-type *C. rodentium* (8). Infections

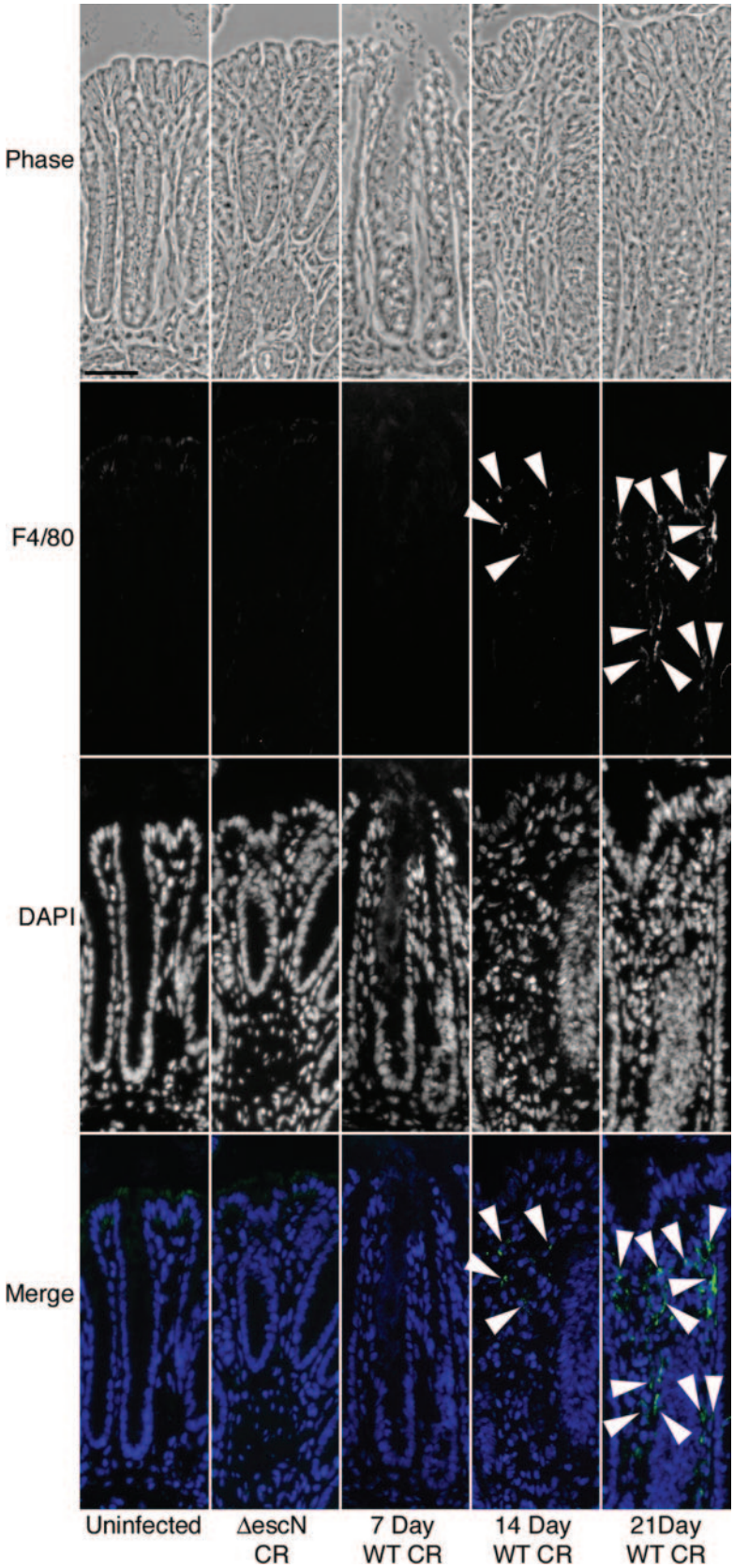
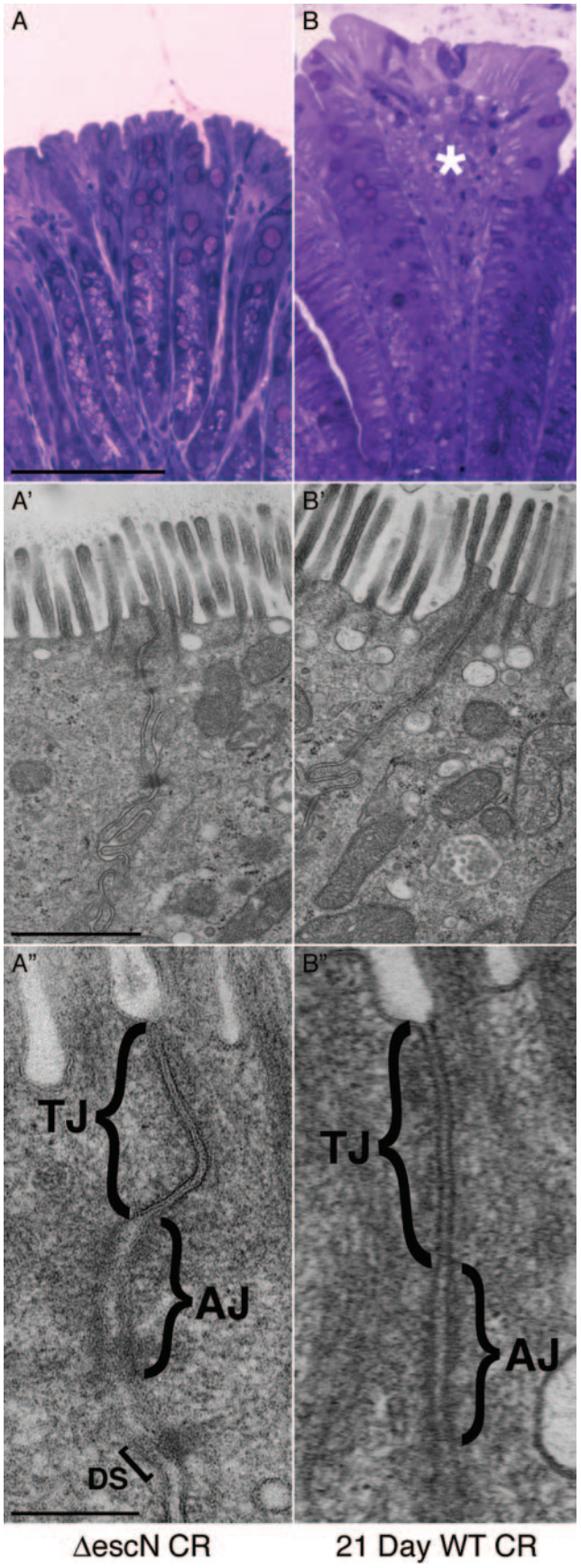


FIG. 3. Paired phase, F4/80 (macrophage marker), and DAPI micrographs of uninfected, Δ*escN C. rodentium* (Δ*escN* CR)-infected, and 7-, 14-, and 21-day wild-type *C. rodentium* (WT CR)-infected tissue. Positively labeled cells are not apparent in the lamina propria of uninfected, Δ*escN C. rodentium*-infected, or 7-day wild-type *C. rodentium*-infected colon sections. Positively labeled cells are present in the lamina propria of 14- and 21-day wild-type *C. rodentium*-infected tissue sections (arrowheads). Minimal nonspecific staining is present in the colonocytes and attached *C. rodentium* cells. Scale bar, 25 μm.



persisted for 7, 14, or 21 days, at which point the mice were euthanized by cervical dislocation. All experiments were repeated at least three times.

Tissue preparation, immunolocalization, and neutrophil staining. Tissue immunolocalization was performed as described previously (17). Briefly, tissue was fixed in 3% paraformaldehyde; 5-μm sections were cut by Wax-It Histology Services, Inc., and treated with 0.2% Triton X-100 in phosphate-buffered saline (PBS; 150 mM NaCl, 5 mM KCl, 0.8 mM KH_2PO_4, 3.2 mM Na_2HPO_4, pH 7.3), and samples were washed extensively in PBS and blocked with 5% normal goat serum in PBS containing 0.05% Tween-20 and 0.1% bovine serum albumin (TPBS-BSA). Primary antibodies consisted of rabbit anti-claudin-1, anti-claudin-3, and anti-claudin-5 antibodies used at a concentration of 0.005 mg/ml (Zymed Laboratories, Inc., California) as well as a previously characterized (47) rat serum anti-*C. rodentium* Tir antibody used at a 1:100 dilution and a rat anti-mouse F4/80 antibody directly conjugated to biotin and used at a concentration of 1.315 μg/ml to 3.85 μg/ml (Serotec) in TPBS-BSA with 1% normal goat serum. These antibodies were incubated on the tissue sections overnight at 4°C. The material was washed extensively with the TPBS-BSA and then incubated for 90 min at 37°C with a goat anti-rabbit secondary antibody conjugated to Alexa 568. The slides were again washed extensively and stained with DAPI (4′,6′-diamidino-2-phenylindole). Coverslips were mounted using Vectashield (Vector Labs, Ontario, Canada). The tissue was visualized using a Zeiss Axiophot microscope.

As controls for claudin-1, -3, and -5 immunolocalization, primary antibodies were replaced with normal rabbit IgG at identical concentrations to the primary antibodies. Specific staining was not detected on normal rabbit IgG-stained control sections (data not shown).

Neutrophils were labeled according to the manufacturer's instructions using a Naphthol AS-D Chloroacetate Esterase kit (Sigma). Tissue was counterstained using hematoxylin.

Electron microscopy and thick sections. Two- to three-millimeter sections of colon were excised from euthanized mice and fixed at room temperature for 2 to 3 h by immersion in a solution consisting of 1.5% paraformaldehyde, 1.5% glutaraldehyde, and 0.1 M sodium cacodylate (pH 7.3). The sections of bowel were cut into smaller pieces (1 to 1.5 mm), washed three times (10 min each wash) in 0.1 M sodium cacodylate buffer (pH 7.3), and then postfixed on ice for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.3). The material was washed three times in distilled water and then stained en bloc in 1% aqueous uranyl acetate. After 1 h, the samples were washed with distilled water, dehydrated through an ascending series of ethyl alcohols, infiltrated through propylene oxide into resin (EPON 812), and then embedded in resin. Thick sections were cut at a 1-μm thickness, mounted on glass slides, stained with toluidine blue, and then photographed on a Zeiss Axiophot microscope. Thin sections were cut at about 700 Å, mounted on grids, stained with uranyl acetate and lead citrate, and photographed on a Philips 300 electron microscope operated at 60 kV.

Tracer experiments. Tracer experiments have been described previously (17). Briefly, EZ-link Sulfo-NHS-Biotin (Pierce Chemical Co., Illinois) was diluted to 2 mg/ml in PBS–1 mM $CaCl_2$ and slowly injected into the distal colon for 3.5 min. Following this, 1 cm of colon, just cranial to the area contacting the needle, was removed and fixed in 3% paraformaldehyde in PBS for 3 h. The tissue was then washed in PBS and sectioned by Wax-It Histology Services, Inc. Tissue sections were incubated with a 1:500 dilution of streptavidin conjugated to Alexa 488 for 30 min at room temperature and imaged using a Zeiss Axiophot microscope. As controls tissue sections that were not pretreated with biotin were incubated with streptavidin to investigate endogenous biotin activity. These controls were negative (data not shown).

FIG. 4. Histologic and ultrastructure micrographs of infected tissue. The 21-day wild-type *C. rodentium* (WT CR)-infected tissue (B and B″) is paired with 7-day Δ*escN C. rodentium* (Δ*escN* CR)-infected mouse tissue (A and A″). Toluidine blue (A and B) and electron micrographs (A′ and B′) of Δ*escN* and wild-type *C. rodentium*-infected tissue are shown. The A″ and B″ images are higher magnifications of the apical junction complexes in the A′ and B′ micrographs. The asterisk indicates a location of cell infiltration in the inflamed epithelium. TJ, tight junction; AJ, adherens junction; DS, desmosome. Scale bars, 50 μm (A), 1.0 μm (A′), and 0.2 μm (A″).

RESULTS

Tight junctions remain morphologically and functionally intact in the presence of A/E bacterially induced inflammation in vivo. To assess what role the inflammatory response has on tight junction disruption caused by A/E pathogens, we infected mice with wild-type *C. rodentium* for 21 days and compared claudin-1, -3, and -5 localization on tissue sections with material from uninfected and 21-day Δ*escN C. rodentium*-infected mice as well as 7-day wild-type *C. rodentium*-infected tissue sections. The 21-day time point was studied because 21 days postinfection, *C. rodentium* is cleared by the animal (25), but significant inflammation persists. The TTSS mutant Δ*escN C. rodentium* is cleared by the animal by about day 7, does not attach to colonocytes, and, consequently, does not cause disease; an animal infected with Δ*escN C. rodentium* is indistinguishable from an uninfected animal (17). For these reasons, we use the Δ*escN C. rodentium* infection as a sham control. Following 21-day infection, localization of claudin-1, -3, and -5 in colonocytes appeared unaltered compared to uninfected or Δ*escN C. rodentium*-infected mice, whereas 7-day infections demonstrated extensive claudin redistribution away from the cell periphery (Fig. 1). The characteristic staining pattern remained along the lateral cell boundaries (Fig. 1, arrowheads). Wild-type *C. rodentium*-infected mice had inflamed colons, as demonstrated by increased cell infiltrate in the lamina propria (Fig. 1). The inflammatory cell infiltrate was confirmed by neutrophil and macrophage (F4/80) staining (Fig. 2 and 3).

We then determined if the unaltered localization of the claudin proteins correlated with unaltered tight junction morphology. Careful comparison of colonic epithelial tight junction ultrastructure in 21-day wild-type *C. rodentium*-infected animals with that in mice infected with Δ*escN C. rodentium* (a sham infection; indistinguishable from uninfected tissue) indicated to us that there were no morphological differences with the tight junctions of 21-day wild-type-infected colonocytes, even though increased cellular infiltrate was observed in the lamina propria of wild-type-infected samples (Fig. 4).

Tight junctions maintain barrier function during A/E pathogen-induced inflammation. Tight junctions in the murine colon were assessed for functional integrity following 21-day infection with *C. rodentium*. The aim of this was to determine if the unaltered localization of claudin proteins corresponds to functionally intact tight junctions. We injected a molecular tracer (biotin) into the distal colon of 21-day wild-type *C. rodentium*-infected mice and compared these results to Δ*escN C. rodentium*-infected mice. We have used this technique previously to demonstrate that tight junctions are disrupted in the presence of wild-type *C. rodentium* at 7 days postinfection, when inflammatory cells are not evident in the lamina propria (17) (Fig. 5).

Following biotin treatment of 21-day wild-type *C. rodentium*-infected animals, the biotin tracer lined the luminal boundary of the colonic epithelium and did not penetrate into the tissue (Fig. 6). This indicates that the tight junctions during the inflammatory events caused by A/E pathogen infection are intact and maintain the epithelial barrier (Fig. 6). No specific tissue labeling was present in controls (data not shown).

Tight junctions are altered by *C. rodentium* intimately attached to colonocytes in vivo. To investigate whether or not the intimate attachment of *C. rodentium* to colonocytes is a prerequisite for tight junction disruption, murine colon sections from mice 14 days after *C. rodentium* infection were labeled with antibodies to claudin-3. Claudin-3 staining was pursued because of the superiority of the antibody over claudin-1 or -5 antibodies used in this study. We previously have shown that

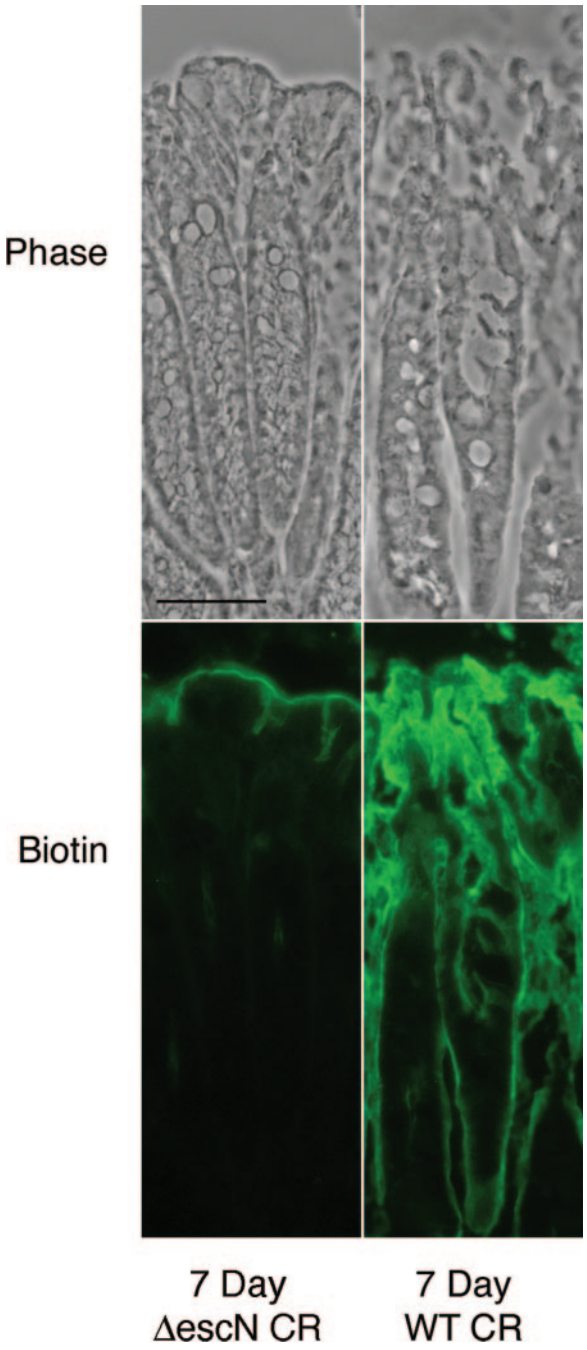


FIG. 5. Barrier permeability micrographs of 7-day *C. rodentium* infections. Phase images of 7-day Δ*escN C. rodentium* (Δ*escN* CR)-infected and 7-day wild-type *C. rodentium* (WT CR)-infected tissue pretreated with a biotin tracer to assess barrier permeability. Biotin is held to the luminal border in Δ*escN C. rodentium*-infected tissue but permeates the epithelium into the lamina propria in 7-day wild-type *C. rodentium*-infected tissue. Scale bar, 50 μm.

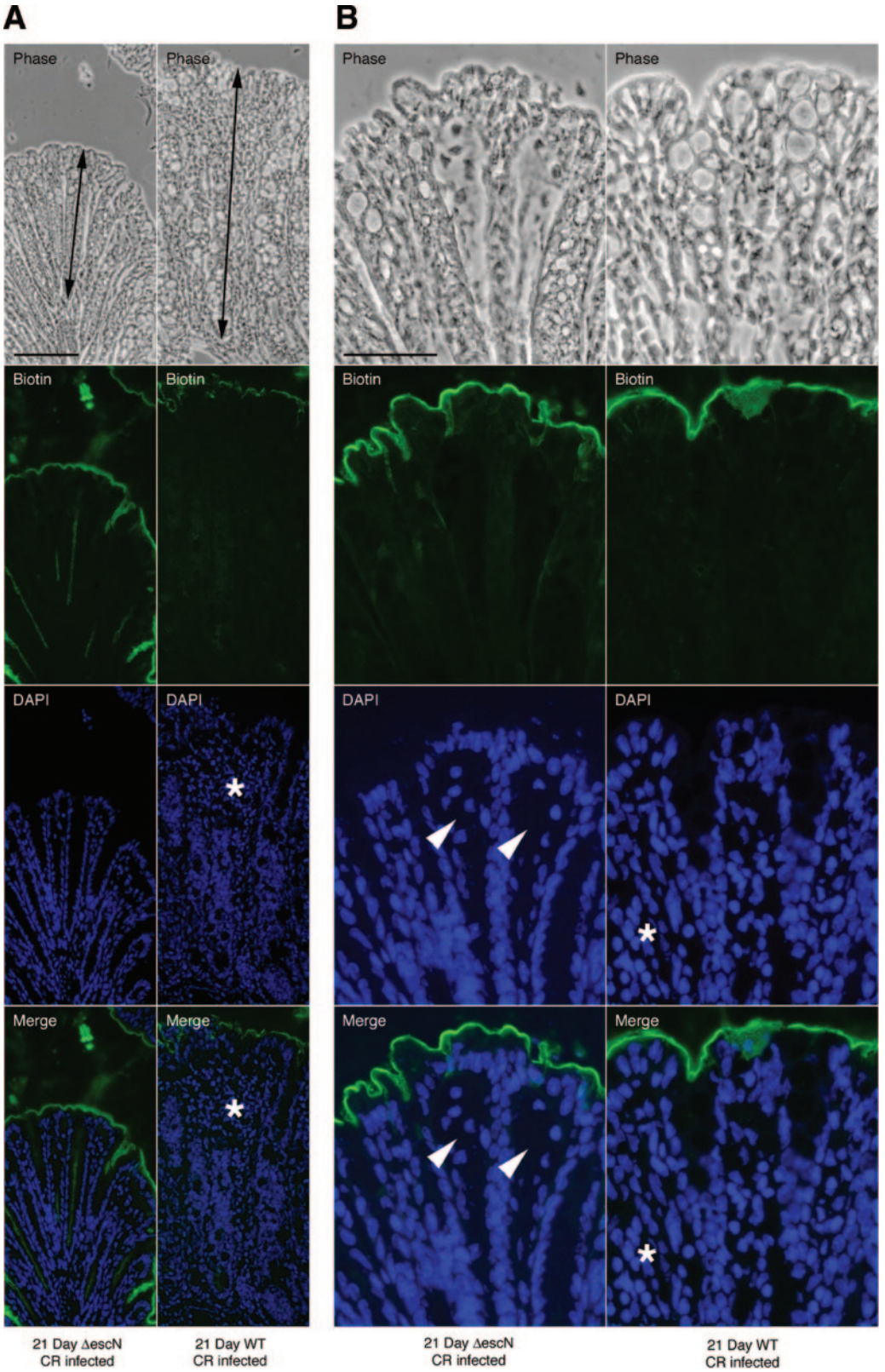


FIG. 6. Barrier permeability micrographs. (A) Phase images of low magnification of 21-day Δ*escN C. rodentium* (Δ*escN* CR)-infected and wild-type *C. rodentium* (WT CR)-infected tissue pretreated with a biotin tracer to assess barrier permeability. Biotin is held to the luminal border in both Δ*escN C. rodentium*-infected and wild-type *C. rodentium*-infected tissue. (B) Higher magnification of biotin- and DAPI-treated murine tissue that was infected with Δ*escN C. rodentium* or wild-type *C. rodentium* for 21 days. Double-headed arrows demonstrate the difference in crypt depth between Δ*escN C. rodentium*-infected and wild-type *C. rodentium*-infected tissue to indicate that the disease phenotype has not resolved at this time point. The asterisks in the DAPI and merged images indicate some of the regions with high levels of cellular infiltrate in the 21-day wild-type *C. rodentium*-infected inflamed epithelium. Arrowheads point to regions with normal cellular load in the lamina propria. Scale bars, 100 μm (low magnification) and 50 μm (high magnification).

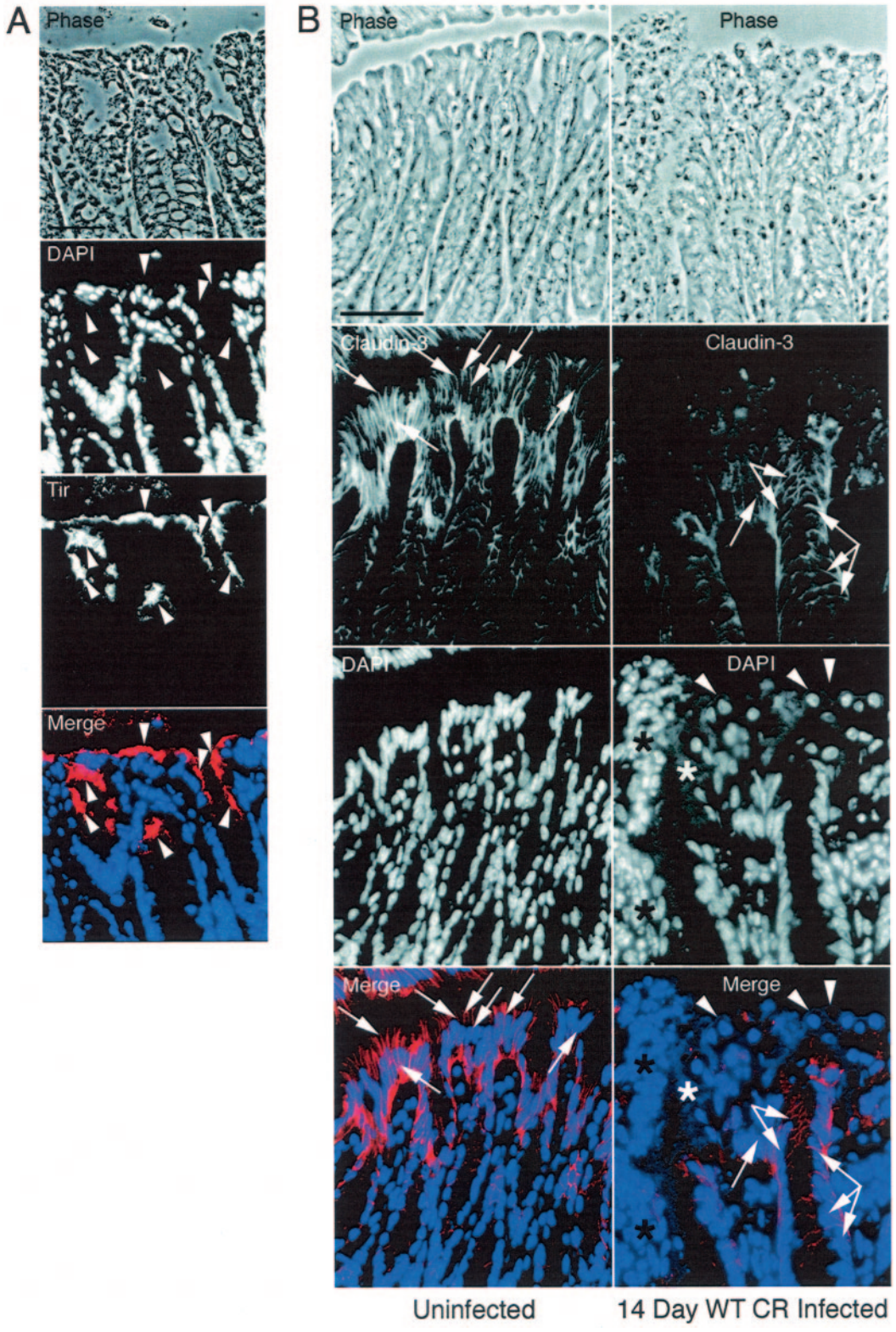


FIG. 7. (A) Paired phase, *C. rodentium* Tir, and DAPI micrographs of infected tissue. Tir and DAPI colocalization demonstrates that bacteria labeled by DAPI in infected tissue sections is *C. rodentium*. Scale bar, 50 μm. (B) Paired phase, claudin-3, and DAPI micrographs of uninfected and 14-day wild-type *C. rodentium* (WT CR)-infected tissue. Arrows indicate regions of the epithelium without bacterial attachment that label the lateral boundaries of colonocytes. Arrowheads and white asterisks identify regions with bacterial colonization and direct attachment to colonocytes. All infected regions have altered localization of claudin-3. The black asterisks in the DAPI and merged images indicate areas with high levels of cellular infiltrate. Scale bar, 50 μm.

the appropriate localization of claudin-3 at the cell boundaries in colonocytes corresponds to intact tight junctions whereas the altered localization of claudin-3 corresponded to functional tight junction deficiencies in the colon (17).

At 14 days after infection, bacterial colonization and inflammation are at high levels. Claudin-3 localization was disrupted only in colonocytes that had intimately attached bacteria (Fig. 7). At areas of the same tissue devoid of bacteria, claudin-3 localization was unaffected (Fig. 7). These results further confirm our findings that tight junction disruption is unaffected by the inflammatory response during A/E bacterial infection and that any tight junction alteration is due to the direct contact of the pathogen to the host's cells (Fig. 7). Additionally, this evidence demonstrates that other host cell mediators likely do not alter tight junctions during in vivo A/E pathogen infections.

DISCUSSION

We have previously demonstrated that uninfected and Δ*escN C. rodentium*-infected mice display indistinguishable claudin phenotypes whereas wild-type *C. rodentium* infections cause overt tight junction disruption between infected colonocytes of mice that are exposed for 7 days. At this time point postinoculum, inflammatory cell infiltrate in the lamina propria of infected mice is not evident. However, 21 days after wild-type *C. rodentium* infection, inflammation persists in the absence of colonized bacteria. The temporal separation of inflammation from A/E bacterial colonization at 21 days provides a model to study the effects of bacterial colonization versus inflammation in vivo. We demonstrate here, morphologically and by the localization of claudin proteins, that tight junctions are unaltered in the presence of inflammatory cells during the inflammatory response caused by *C. rodentium* infection. These findings correspond to a functionally intact epithelial barrier.

Thus far, only one study has explored the role of A/E pathogen-induced inflammation on tight junctions (39). In that study, the MAP or ERK kinase inhibitor PD-98059 was used on T84 cells that were subsequently infected with EPEC. Although these kinases are capable of activating inflammatory cascades, they can also have direct effects on tight junctions (2, 21, 49) and can influence numerous other events such as cell proliferation, protection against apoptosis, and cell survival (5, 20, 29, 33, 44). Results from the Savkovic (39) study found equivalent decreases in TER caused by EPEC on cultured T84 monolayers in the presence or absence of PD-98059. This suggested to the authors that the relationship of cytokine release to tight junction alteration was not present because TER was still decreased in cells infected in the presence PD-98059. Although this study used an inhibitor that can directly act on tight junctions (2, 21, 49), the effects of both MAP and ERK in vivo are an area that should still be pursued in vivo.

Recently, a second mouse model has emerged for the study of A/E pathogen infections in vivo (40). In this model, the human A/E pathogen EPEC was used to infect mice. During infection, inflammation is minimal, and bacterial counts have been reported in various parts of the intestine at about 10^4 CFU (40). These counts correspond to those of *C. rodentium* lacking a functional type III secretion system (Δ*escN*), which does not colonize the colon, and signify extremely low levels of colonization of EPEC in mice. A recent study by this group using the EPEC murine model (42) suggested that tight junctions can be disrupted in vivo in a TTSS-dependent manner by EspF, but the presented photographic evidence indicated the absence of any bacteria intimately attached to the cells. Although this suggested that potential mediators might be released from infected host cells to influence tight junctions in noninfected cells, our evidence demonstrates that intimate contact of the bacteria to cells is essential for barrier alteration to occur.

Here, we present evidence that the inflammatory response caused by *C. rodentium* has no discernible effect on morphology or the barrier function of tight junctions. We also demonstrate that the alteration of claudin-3 localization in the infected murine colon is completely dependent on the intimate attachment of the pathogen to the colonic enterocytes. Our evidence further accentuates the importance of using relevant in vivo studies and infection models to assess the role of pathogenic infections on tissues.

ACKNOWLEDGMENTS

The authors thank Wanyin Deng, Bruce Vallance, and Bryan Coburn for their critical review of the manuscript and Guntram Grassel for his technical assistance.

J.A.G. is a CAG/CIHR/AstraZeneca and MSFHR Postdoctoral Fellow. B.B.F. is a Howard Hughes International Research Scholar, a CIHR Distinguished Investigator, and the UBC Peter Wall Distinguished Professor. This study was funded through operating grants from the CIHR and the HHMI.

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Editor: A. D. O'Brien