

## Pretreatment of Epithelial Cells with Rifaximin Alters Bacterial Attachment and Internalization Profiles<sup>∇</sup>

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**Rifaximin is a poorly absorbed semisynthetic antibiotic derivative of rifampin licensed for use in the treatment of traveler's diarrhea. Rifaximin reduces the symptoms of enteric infection, often without pathogen eradication and with limited effects on intestinal flora. Epithelial cells (HEp-2 [laryngeal], HCT-8 [ileocecal], A549 [lung], and HeLa [cervical]) were pretreated with rifaximin (or control antibiotics) prior to the addition of enteroaggregative *Escherichia coli* (EAEC). EAEC adherence was significantly reduced following rifaximin pretreatment compared to pretreatment with rifampin or doxycycline for three of the four cell lines tested. The rifaximin-mediated changes to epithelial cells were explored further by testing the attachment and internalization of either *Bacillus anthracis* or *Shigella sonnei* into A549 or HeLa cells, respectively. The attachment and internalization of *B. anthracis* were significantly reduced following rifaximin pretreatment. In contrast, neither the attachment nor the internalization of *S. sonnei* was affected by rifaximin pretreatment of HeLa cells, suggesting that rifaximin-mediated modulation of host cell physiology affected bacteria utilizing distinct attachment/internalization mechanisms differently. In addition, rifaximin pretreatment of HEp-2 cells led to reduced concentrations of inflammatory cytokines from uninfected cells. The study provides evidence that rifaximin-mediated changes in epithelial cell physiology are associated with changes in bacterial attachment/internalization and reduced inflammatory cytokine release.**

Rifaximin is a semisynthetic rifamycin derivative that has been safely and successfully used to treat and prevent traveler's diarrhea (6, 7, 13, 26). Rifaximin's additional pyridoimidazole ring makes it virtually nonabsorbable (<0.4%), leading to high concentrations of the drug in feces after oral administration (15). Rifaximin has also been used successfully in the treatment of irritable bowel syndrome (9), infectious diarrhea (7, 8), small bowel bacterial overgrowth (29), hepatic encephalopathy (18, 19), pouchitis (7), and fulminant ulcerative colitis (12). The mechanism(s) of action of rifaximin in these diverse conditions remains poorly defined. Rifaximin has been shown to have effects on gene-regulatory pathways in mammalian cells (13, 14), suggesting that the drug may have effects on mammalian cells independent of its antimicrobial properties. Furthermore, rifaximin is a unique antibiotic in several ways: (i) bacterial pathogens show a low potential for developing resistance during rifaximin therapy, in contrast to the situation with the structurally related drug rifampin (4); (ii) following oral administration, the drug has little effect on Gram-negative coliforms in the gut or on Gram-positive enterococcal flora (7, 8); (iii) the drug ameliorates diarrheal disease symptoms, often without significant alteration to the intestinal pathogen burden or the counts of intestinal flora (1, 7, 8), even though rifaximin concentrations can reach ~8,000 µg/g of stool after 3 days of treatment (15). These observations suggest the possibility that some of the positive results of rifaximin treatment could be due

both to direct drug effects on the pathogen and to an effect on intestinal cells, rendering them resistant to bacterial colonization/internalization.

Since many pathogenic bacteria need to attach to or invade host epithelial cells as a first step in the disease process, we investigated the possibility that rifaximin could affect bacterial attachment. Since enteroaggregative *Escherichia coli* (EAEC) is phenotypically characterized by its "stacked-brick" adherence pattern on HEp-2 cells, we tested the adherence of EAEC strain O42 to rifaximin-pretreated HEp-2 (laryngeal) cells in addition to examining its attachment to HCT-8 (ileocecal) A549 (lung), or HeLa (cervical) cells pretreated with either rifaximin, rifampin, doxycycline, or acetone (solvent control). The data presented in this report demonstrate that rifaximin pretreatment, but not pretreatment with controls, significantly decreased EAEC attachment to HEp-2, A549, and HeLa cells, suggesting that some of the beneficial effects of this drug are due to modifications to the host cell epithelium and that different cell types respond differently to rifaximin treatment. Rifaximin-mediated changes to host cells were also examined in the context of different bacteria, i.e., we assessed the adherence and internalization of either *Bacillus anthracis* or *Shigella sonnei* into either A549 cells or HeLa cells, respectively (24, 25). These studies demonstrated that rifaximin pretreatment had no effect on *S. sonnei* adherence or internalization but significantly altered these parameters for *B. anthracis*. These data suggest that rifaximin-mediated changes to epithelial cell physiology do not affect all bacteria, i.e., the adherence mechanisms utilized by EAEC are very different from those used by *B. anthracis*, and the type III secretory system utilized by *S. sonnei* to gain access to epithelial cells is not found in *B. anthracis* (23).

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## MATERIALS AND METHODS

**Bacterial strains and cell lines.** EAEC strain O42 (provided by J. Nataro, University of Maryland School of Medicine, Baltimore, MD) (20), *S. sonnei* (a clinical isolate obtained from a patient in India by our laboratory in 2008), and *B. anthracis* Sterne strain 7702 (provided by T. M. Koehler, University of Texas Health Science Center, Houston, TX) were used in this study.

HEp-2 (a human larynx squamous cell carcinoma) (5, 12), HCT-8 (a human intestinal adenocarcinoma cell line) (11), A549 (a human lung adenocarcinoma epithelial cell line) (24), and HeLa (a cervical cancer cell line) were obtained from the American Type Culture Collection. These cell lines were used in this study and were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO<sub>2</sub>. No antibiotics were used in the preparation of the medium.

**HEp-2 cell assay.** The HEp-2 cell attachment assay was carried out as described previously (11), except that confluent monolayers were used instead of cells grown to 50 to 80% confluence unless otherwise specified. HEp-2 cells were grown in Lab-Tek II (Nunc, Rochester, NY) chambered slides and were incubated in the presence of antibiotics or the appropriate controls for 4, 8, 16, 18, and 24 h. At the end of the antibiotic incubation period, chambers were washed three times with PBS (phosphate-buffered saline, pH 7.4) prior to the addition of EAEC ( $7 \times 10^6$  to  $1 \times 10^7$  bacteria in 1 ml DMEM, 10% FBS, and 1% D-mannose) (2, 9) that had been cultured at 37°C overnight in Trypticase soy broth (Difco, Lawrence, KS) with 1% D-mannose (Sigma, St. Louis, MO). After a 3-h incubation at 37°C, the attachment of *E. coli* O42 to epithelial cells was visualized microscopically following Wright-Giemsa staining (Protocol Hema 3; Fisher Diagnostics, Middletown, VA) using an Olympus BX60 inverted microscope attached to an Olympus C-5060 digital camera.

**Quantification of bacterial adherence and internalization.** HEp-2, HCT-8, A549, or HeLa cells were grown to confluence in 24-well plates (Corning Inc., Corning, NY) and incubated with the respective antibiotics or controls. Twenty-four hours later, the supernatants were removed, and the wells were washed gently three times with 1 ml PBS prior to the addition of either EAEC strain O42 (2, 9) or *S. sonnei* ( $7 \times 10^6$  to  $1 \times 10^7$  in 1 ml DMEM, 10% FBS, 1% D-mannose) grown at 37°C overnight in Trypticase soy broth with 1% D-mannose, or *B. anthracis* spores ( $4.8 \times 10^5$ ) prepared from Sterne strain 7702 in phage assay (PA) medium as described previously (10, 24, 25).

For the quantification of bacterial adherence, EAEC O42 was incubated in the presence of the respective antibiotic-pretreated cell lines, and *S. sonnei* was incubated in the presence of antibiotic-pretreated HeLa cells, in 24-well plates for 3 h at 37°C. Following this incubation, the supernatants were removed, and the wells were washed three times with 1 ml sterile PBS. Distilled water (1 ml) was then added to the wells, and the cells were removed by pipetting, diluted in sterile PBS, and plated on Luria-Bertani (LB) agar plates for quantification by counting of CFU 16 h later. For *B. anthracis* attachment, A549 cells were grown to confluence in 24-well tissue culture plates and pretreated for 24 h with antibiotics as described above. The cells were washed three times with PBS to remove antibiotics and were then infected with Sterne strain 7702 spores for 1 h at 37°C in a humidified chamber with 5% CO<sub>2</sub>. Unbound spores were removed by three washes with PBS, and the A549 cells were removed and plated on LB agar plates as described above for CFU determinations.

For the internalization assays, *S. sonnei* or *B. anthracis* was incubated with antibiotic-treated HeLa or A549 cells, respectively, as described above. After the bacteria were incubated with the respective cell lines and the wells were washed, the epithelial cells were further incubated with a medium containing gentamicin (100 µg/ml) for 1 h to kill noninternalized bacteria, washed with PBS, lysed in distilled water, and plated for quantification as described above.

In one experiment, the number of EAEC bacteria in the supernatants following the 3-h incubation with HEp-2 cells treated with various antibiotics or controls was determined. Supernatants were collected, and the wells were washed three times with 200 µl sterile PBS. The PBS washes and the supernatants were combined, centrifuged, and resuspended in 1 ml sterile PBS; the suspension was then serially diluted and plated on LB agar plates for quantification as described above.

The CFU data are means  $\pm$  standard errors (SE) from triplicate wells for bacteria that adhered or were internalized and are expressed as percentages of the total bacteria added to the respective treatment wells. Statistical differences in bacterial counts were determined using Student's *t* test.

**Antibiotics.** Rifaximin (Salix Pharmaceuticals Inc., Morrisville, NC), rifampin (Sigma), doxycycline (Sigma), or gentamicin (Sigma) were tested in attachment/internalization assays. Rifaximin was dissolved in acetone and rifampin, and doxycycline and gentamicin were dissolved in water (1-µg/µl stocks of each were prepared). Antibiotics were prepared fresh at the start of each experiment.

Antibiotics and acetone were sterile filtered with a 0.22-µm-pore-size syringe filter before use. Antibiotics were incubated with HEp-2 cells for various times at 8, 32, or 64 µg/ml and with HCT-8, A549, or HeLa cells for 24 h at 32 or 64 µg/ml. Acetone was administered at a volume equivalent to the volume used to deliver each respective antibiotic concentration as a negative control. The concentrations tested were based on the MICs of rifaximin and rifampin. The highest dose of rifaximin selected (64 µg/ml) corresponded to the MIC<sub>50</sub> previously established for EAEC in our laboratory (14).

**Supernatant dialysis.** HEp-2 cells were cultured in T-175 flasks as described above. When the cells reached confluence, the medium was removed and replaced with fresh medium containing either no antibiotics, rifaximin, rifampin, doxycycline, or acetone (64 µg/ml or acetone at the same volume used to deliver the antibiotics). After 24 h, the medium was collected, loaded into regenerated cellulose dialysis tubing (cutoff, 6 to 8 kDa; Spectra/Por, Rancho Dominguez, CA), and dialyzed against 4 liters of PBS changed four times. Each dialysis step lasted at least 6 h and was carried out at 4°C. Dialyzed supernatants were either used immediately in adherence assays or stored at -20°C until use.

The effect of supernatants on EAEC adherence was determined by mixing EAEC with the dialyzed media from the different antibiotic (or control) treatment groups and adding the bacteria to HEp-2 cells cultured in chambered slides or to chambered slides with no HEp-2 cells. Adherence was visualized microscopically as described above.

**Cytokine arrays.** HEp-2 cells were plated on chambered wells and grown to confluence. The medium was replaced with fresh medium containing either antibiotics (64 µg/ml), a diluent control (64 µl) (acetone), or medium with no antibiotics. Supernatants were collected 24 h later and were used to probe a cytokine array membrane (Panomics, Fremont, CA) that supported the detection of Apo/Fas, cytotoxic T-lymphocyte-associated antigen (CTLA), eotaxin, granulocyte-monocyte colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), gamma interferon (IFN-γ)-inducible protein 10 (IP-10), leptin, monocyte inflammatory protein 1α (MIP1α), MIP1β, MIP4, MIP5, matrix metalloproteinase 3 (MMP3), RANTES (regulated on activation, normal T-expressed and secreted), transforming growth factor β (TGF-β), IFN-γ, tumor necrosis factor alpha (TNF-α), TNF receptor 1 (TNFR1), TNFR2, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), vascular endothelial growth factor (VEGF), interleukin-1α (IL-1α), IL-1β, IL-Rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-8, IL-10, IL-12 (p40), IL-15, and IL-17 as described by the manufacturer. The presence of the respective supernatant proteins was visualized by exposing and then developing Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, United Kingdom) film. Blots probed with the respective supernatants were analyzed at the same time and developed simultaneously on the same Hyperfilm.

## RESULTS

**Effect of rifaximin on EAEC adherence.** The effect of rifaximin on bacterial attachment was first examined using the standard HEp-2 cell adherence assay commonly carried out to phenotypically define EAEC isolates based on their stacked-brick adherence pattern (8, 15). HEp-2 cells were grown to confluence and were then incubated with rifaximin, rifampin, doxycycline, or acetone. After 24 h, the wells were washed prior to the addition of the EAEC strain O42. EAEC O42 adhered in the traditional stacked-brick formation to untreated HEp-2 cells (Fig. 1A) or HEp-2 cells treated with rifampin, doxycycline, or acetone (data not shown); however, the level of adherence to rifaximin-pretreated HEp-2 cells was greatly reduced (Fig. 1B). These effects were time and concentration dependent (data not shown); however, since pretreatment with 64 µg/ml rifaximin for 24 h had the greatest effects on bacterial attachment, this dose was used in subsequent experiments.

EAEC adherence to HEp-2 cells following pretreatment with the various antibiotics or controls was quantified. The level of EAEC adherence to rifaximin-pretreated cells was significantly lower than the level of adherence to untreated cells or to cells treated with either rifampin, acetone (Fig. 1C),

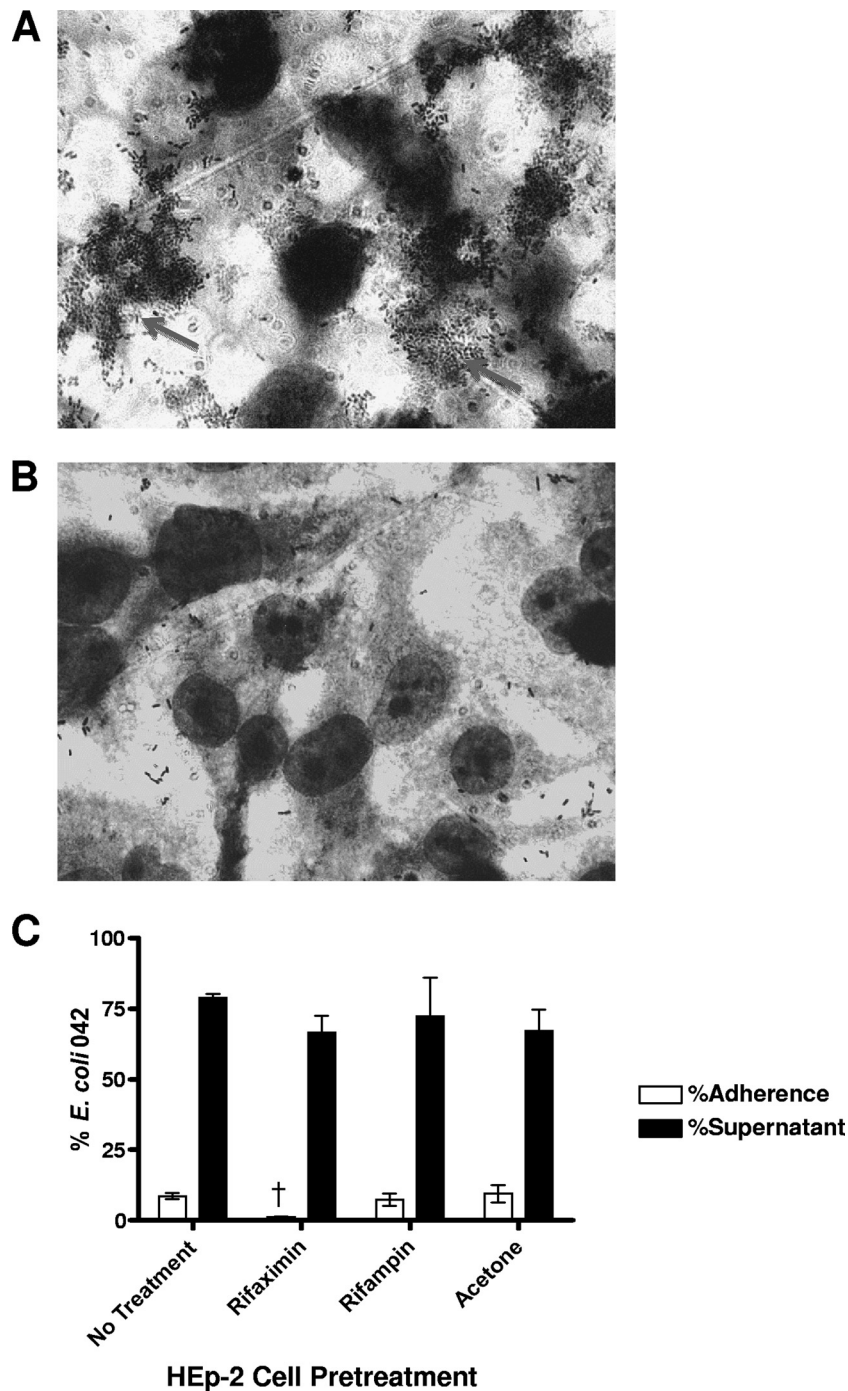


FIG. 1. EAEC attachment following pretreatment with rifaximin. (A) Untreated HEp-2 cells incubated with  $7 \times 10^6$  EAEC bacteria. The arrows indicate EAEC attaching to the HEp-2 cell and slide surfaces (magnification,  $\times 100$ ). (B) HEp-2 cells pretreated for 24 h with rifaximin (32  $\mu\text{g/ml}$ ), washed, and then incubated with  $7 \times 10^6$  EAEC bacteria (magnification,  $\times 100$ ). The arrow indicates cells attaching to the slide surface and leading edge of HEp-2 cells. (C) Concentrations of EAEC in the supernatant or attached to the cell surface. HEp-2 cells grown to confluence in 24-well plates were pretreated with rifaximin, acetone, or rifampin, or were left untreated, for 24 h; then they were washed as described in Materials and Methods and were incubated with EAEC for 3 h in triplicate. To determine the CFU of EAEC in the supernatant, supernatants from respective wells were collected, and the wells were washed three times with PBS (200  $\mu\text{l}$  each time). The supernatants and the washes were combined, centrifuged, resuspended in 1 ml PBS, serially diluted, and plated onto LB agar plates. To determine the number of EAEC bacteria attached, HEp-2 cells were trypsinized, washed, and plated onto LB plates as described above. The data are expressed as mean percentages of the total bacteria added to the wells that were found in the supernatant or attached to the cell surface. Error bars, SE. CFU counts in the supernatants or cell fractions of triplicate wells demonstrated reduced EAEC adherence to HEp-2 cells pretreated with rifaximin. †,  $P < 0.05$  by an unpaired Student  $t$  test.

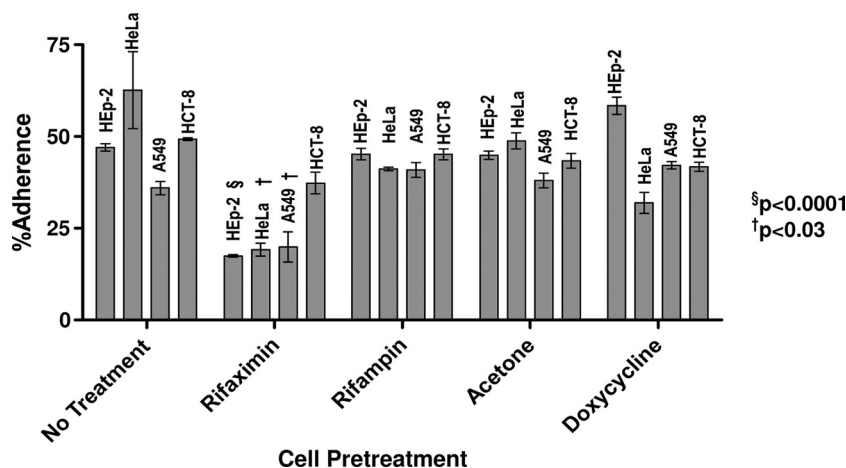


FIG. 2. Rifaximin pretreatment reduced EAEC binding to epithelial cells. Confluent HEp-2, HeLa, A549, or HCT-8 cells were cultured for 24 h either in medium only or in medium containing 64  $\mu\text{g/ml}$  of either rifaximin, rifampin, acetone (64  $\mu\text{l}$ ), or doxycycline in triplicate. The wells were washed, and  $1 \times 10^7$  EAEC bacteria were added and incubated for 3 h. Then the wells were washed three times with 1 ml of PBS (each wash), and the cells were lysed by addition of 1 ml of sterile distilled water. Serial dilutions were then made in sterile PBS and plated onto LB agar plates to determine the number of CFU per well. The data are expressed as the percentage of the total number of bacteria added to the wells that adhered to the cells. *P* values, determined by an unpaired Student *t* test, were  $<0.0001$  (§) and  $<0.03$  (†).

or doxycycline (not shown). No differences in bacterial counts were observed in the supernatants (Fig. 1C).

To determine if the observed reduction in EAEC adherence following rifaximin pretreatment of HEp-2 cells extended to other cell types or bacteria, we examined EAEC O42 adherence to three additional cell lines. We examined the effects of rifaximin pretreatment on adherence and internalization using two vastly distinct pathogens: *B. anthracis* and *S. sonnei*.

EAEC adherence to HEp-2, HCT-8, A549, and HeLa cells was measured by carrying out adherence assays as described above. The respective cell lines were grown to confluence in 24-well plates, and fresh medium containing no antibiotics or 64  $\mu\text{g}$  of either rifaximin, rifampin, doxycycline, or acetone (64  $\mu\text{l}$ ) was added for 24 h. Rifaximin pretreatment significantly reduced EAEC adherence to HEp-2, HeLa, and A549 cells. Slightly fewer bacteria adhered to rifaximin-pretreated HCT-8 cells, but this difference was not significant, suggesting differences in cellular susceptibility to rifaximin (Fig. 2).

We measured the adherence and internalization of *B. anthracis* and *S. sonnei* to determine if rifaximin pretreatment affected these processes in different bacteria. *B. anthracis* is a Gram-positive spore-forming bacteria that is the causative agent of anthrax. Humans become infected via spore entry into the lungs or gut or via skin abrasions; infections can result in sepsis and secondary manifestations, including meningitis and potentially death. Recently, the laboratory of Y. Xu established a model of adherence and internalization (using the gentamicin protection assay) for *B. anthracis* Sterne strain 7702 in A549 lung epithelial cells that was modified in this study to examine the role of rifaximin in these processes (24, 25). *S. sonnei* is a Gram-negative member of the family *Enterobacteriaceae* that causes disease in humans by invading and replicating in cells lining the colonic mucosa. Of the four *Shigella* species, *S. sonnei* is the most common cause of shigellosis in developed countries (22).

Following incubations with the respective antibiotics (or controls), significant reductions in both the adherence and the

internalization of *B. anthracis* were observed in rifaximin-pretreated A549 cells compared to those for the other treatment groups, including an internal gentamicin-only treatment group (Fig. 3A and B). In contrast, rifaximin pretreatment of HeLa cells had no effect on *S. sonnei* attachment or internalization compared to those for the other treatment groups under the conditions examined (Fig. 3C and D).

**Effect of HEp-2 supernatants on EAEC adherence.** Because EAEC adherence to HEp-2 cells in the stacked-brick formation is the gold standard for defining EAEC isolates, we next attempted to determine if the observed reduction in EAEC adherence following rifaximin pretreatment was due to rifaximin-mediated alterations in HEp-2 cell physiology that could be conferred via the supernatants of treated cells. To this end, HEp-2 cells were grown in a medium containing 64  $\mu\text{g/ml}$  of either rifaximin, rifampin, or doxycycline (or acetone [64  $\mu\text{l/ml}$ ]) for 24 h. Supernatants were then collected and dialyzed extensively using 6- to 8-kDa-cutoff dialysis tubing to remove any trace of the antibiotics used in the respective treatment groups. This ensured that when the dialyzed media corresponding to the respective antibiotic treatment groups were mixed with EAEC in order to examine the effects of the supernatants on adherence, any changes observed would not be due to antibiotics in the medium. After dialysis, the supernatants were sterile filtered and mixed with EAEC, and adherence to chambered slides with or without HEp-2 cells (50% confluent) was examined. After a 3-h incubation, the slides were washed and stained as described above. EAEC that was mixed with dialyzed supernatants from HEp-2 cells only, i.e., with no antibiotic treatment (Fig. 4A and C), or with dialyzed supernatants from HEp-2 cells pretreated with either rifampin (Fig. 4B), doxycycline, or acetone (data not shown), adhered to chambers independently of the presence of HEp-2 cells (Fig. 4A and B, with HEp-2 cells; Fig. 4C, without HEp-2 cells). EAEC mixed with PBS and added directly to chambers for 3 h adhered in a fashion indistinguishable from the adher-

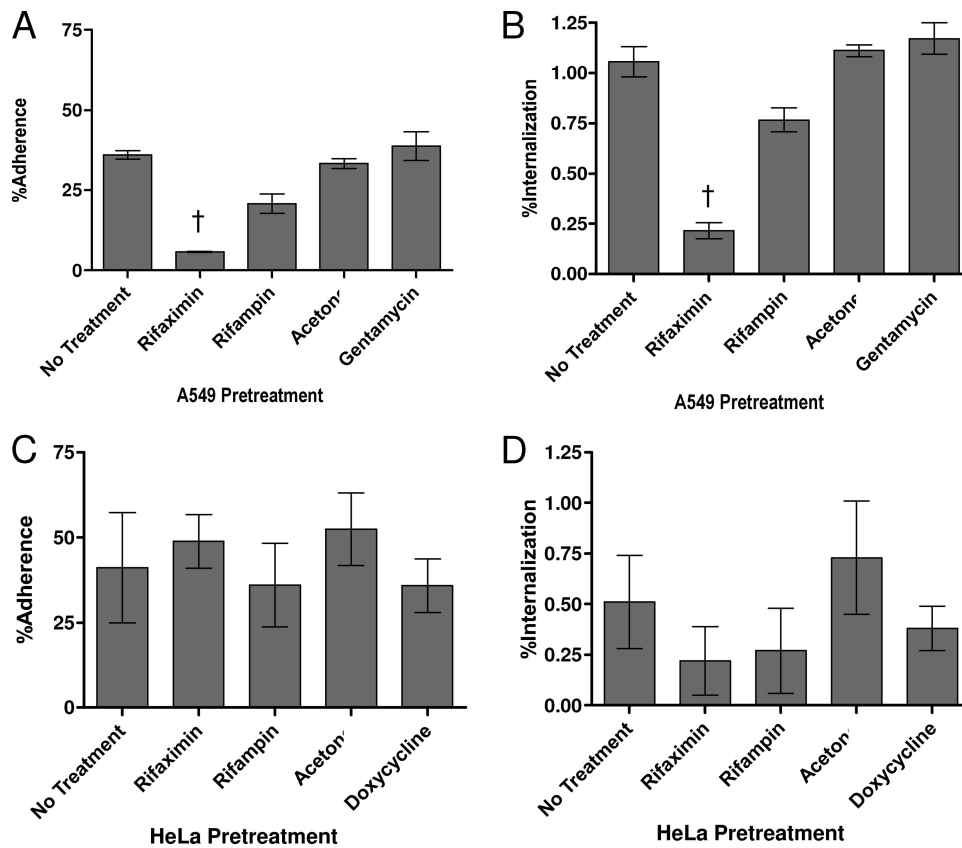


FIG. 3. Effects of rifaximin pretreatment on the adherence and internalization of *B. anthracis* (A and B) or *S. sonnei* (C and D). *B. anthracis* spores ( $4.8 \times 10^5$ ) or *S. sonnei* bacteria ( $1 \times 10^7$ ) were cocultured in wells of 24-well plates containing either A549 or HeLa cells, respectively. For adherence assays, the respective bacteria were incubated for 1 h; the wells were washed; and the cells were plated onto LB agar plates. For internalization, an additional 1-h incubation with gentamicin (100  $\mu\text{g/ml}$ ) was carried out to kill any noninternalized bacteria. The wells were then washed, and the cells were plated onto LB agar plates. The data are expressed as the mean percentages of the total cells added that adhered or were internalized. Error bars, SE. The experiment was repeated twice and with similar results. <sup>†</sup>,  $P < 0.002$  by an unpaired Student *t* test.

ence pattern visible in Fig. 4A to C (data not shown), suggesting that the bacterial adherence pattern was not affected by the medium/buffer used in the adherence assay. In contrast, EAEC that was mixed with dialyzed supernatants from rifaximin-treated HEp-2 cells adhered poorly to chambered wells in the presence or absence of HEp-2 cells (Fig. 4D and E, respectively).

**Cytokine arrays.** Since the supernatants collected from rifaximin-treated HEp-2 cells had the capacity to confer changes in EAEC adherence, we defined the cytokine profiles of the respective supernatants incubated for 24 h alone (Fig. 5A) or pretreated (24 h) with either rifampin (Fig. 5B) or rifaximin (Fig. 4C). GM-CSF, MIP4, MIP5, MMP3, RANTES, TGF- $\beta$ , IFN- $\gamma$ , TNFRI, TNFRII, VCAM-1, VEGF, IL-4, IL-6, IL-8, IL-12 (p40), and IL-15 could be found in the supernatants of untreated (Fig. 5A), doxycycline-treated, or acetone-treated (data not shown) cells. Rifampin-treated cell supernatants had the same profile, except that MIP5 was not detected (Fig. 5B). In contrast, supernatants analyzed from HEp-2 cells cultured in the presence of rifaximin contained detectable levels of RANTES and IL-4 only (Fig. 5C).

## DISCUSSION

Rifaximin is a poorly absorbed antibiotic that effectively treats traveler's diarrhea and has been used in the treatment of *Clostridium difficile*-diarrhea (3, 4, 6, 10, 16). How rifaximin treatment ameliorates disease symptoms remains poorly understood. Although it has been shown to have both bactericidal and bacteriostatic properties that account for some of the antimicrobial benefits derived from rifaximin treatment, one of the most intriguing aspects of this drug is that it can shorten the duration of bacterial diarrhea without invariably eradicating enteropathogen infections and with minimal effects on the colonic bacterial flora (1), suggesting additional mechanisms of action. Although infections with different pathogens can result in diarrheal disease symptoms, the pathophysiological mechanisms employed by these pathogens can differ. Even among diarrheagenic *E. coli* strains, the nature of the microbe-mucosa interactions differs greatly, e.g., for enterotoxigenic, enterohemorrhagic, enteropathogenic, enteroinvasive, and enteroaggregative *E. coli* (21). Alterations in the interactions of the host cells with the pathogen could affect the disease presentation caused by these organisms differently, i.e., rifaximin may alter

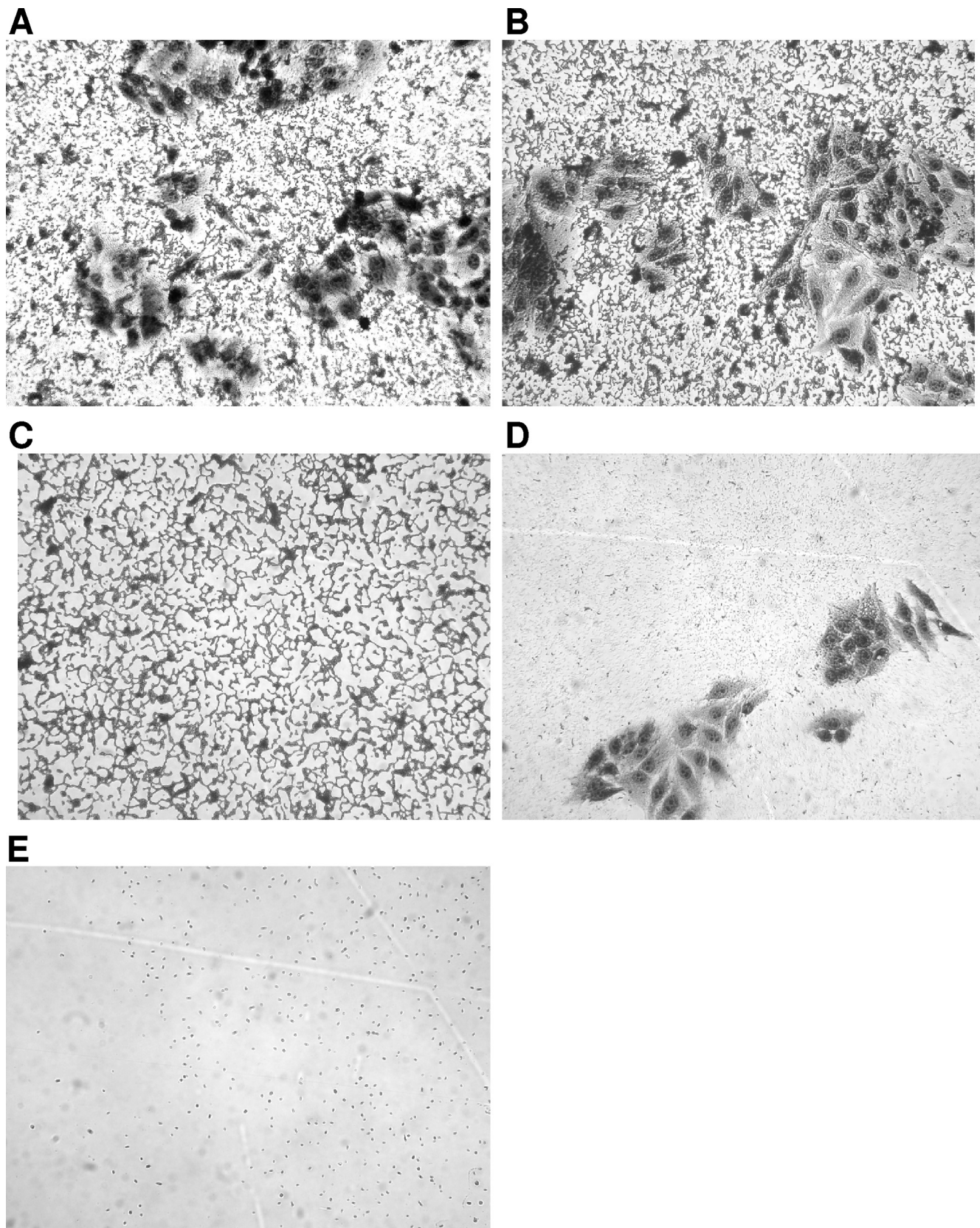


FIG. 4. Effects of dialyzed supernatants on EAEC adherence. EAEC adherence was assessed by adding 750  $\mu$ l of an EAEC solution ( $7 \times 10^6$  bacteria/ml) mixed with 750  $\mu$ l of either dialyzed conditioned medium only (A and C), dialyzed rifampin-conditioned medium (B), or dialyzed rifaximin-conditioned medium (D and E). EAEC adherence was monitored in the presence (A, B, and D) or absence (C and E) of HEp-2 cells. After a 3-h incubation, the chambers were washed and examined microscopically (magnification,  $\times 40$ ) following Wright-Giemsa staining. These experiments demonstrated that a factor (or the lack thereof) in the supernatant of rifaximin-treated cells affected EAEC adherence. This experiment was repeated twice with similar results.

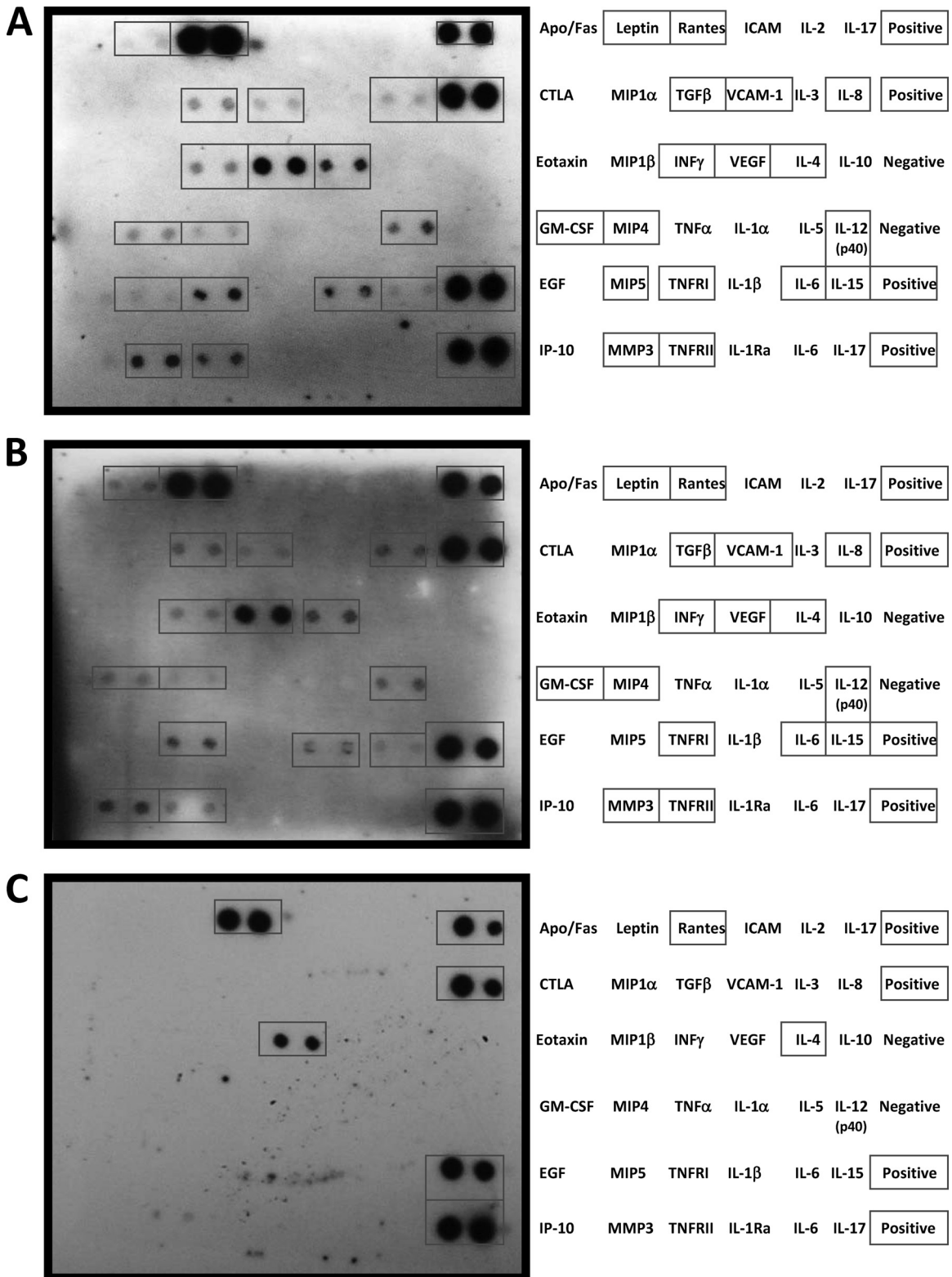


FIG. 5. Supernatant cytokine profile analysis. Supernatants from HEP-2 cells cultured for 24 h without antibiotics (A), in the presence of rifampin (B), or in the presence of rifaximin (C) were analyzed using a Panomics cytokine array. Each membrane array is designed to detect as many as 36 different cytokines (indicated to the right) in duplicate, together with positive and negative internal controls. The blots shown were incubated with the respective supernatants at the same time and were developed on the same film simultaneously. Supernatants from HEP-2 cells treated with doxycycline or acetone were also analyzed (data not shown). This experiment was carried out three times with similar results, demonstrating that rifaximin reduced the expression of various proinflammatory cytokines.

the bacterial adherence of organisms that share adherence or internalization strategies. Reduced mucosal attachment may at least partially explain the beneficial effects of rifaximin when used to prevent traveler's diarrhea caused by diarrheagenic *E. coli* strains (8). The observation that rifaximin is more active against organisms that require interaction with the gut mucosa may further explain why nonpathogenic or normal colonic flora typically not "associated" with the intestinal lining are less affected by this drug and why symptoms associated with EAEC infections are ameliorated without necessarily eliminating the pathogen (1).

In this report we demonstrated that rifaximin pretreatment decreased bacterial adherence to HEp-2 cells without affecting EAEC viability, since equal numbers of bacteria were recoverable from the supernatants of all treatment groups. In contrast, the number of bacteria that adhered to confluent HEp-2 cells pretreated with rifaximin was significantly lower than the number of EAEC bacteria recoverable from cells treated with control antibiotics or left untreated. The fact that there was not an increase in the number of EAEC bacteria in the supernatants of rifaximin-treated cells (since fewer bacteria were recovered from the cell fraction) can be explained as a function of the EAEC cell density added to the cell cultures, i.e., the EAEC bacteria added for the 3-h incubation were grown overnight and were in stationary phase at the time of incubation. During the short time of the adherence assay, significant differences in bacterial growth in the supernatants were not likely to be observed.

Although EAEC adherence to HEp-2, A549, and HeLa cells was significantly reduced following rifaximin pretreatment, little or no change in adherence to HCT-8 cells pretreated with rifaximin was observed, suggesting that different cell lines respond differently to, or have different thresholds of sensitivity to, this compound. Although the nature of the cellular changes affecting EAEC adherence remains undefined, the differences in the adherence and internalization of *B. anthracis* (but not in those of *S. sonnei*) due to rifaximin pretreatment suggested that specific changes to cellular physiology were induced by rifaximin, since these bacteria utilize significantly different adherence/internalization mechanisms. Although we are not suggesting that rifaximin be used for the treatment of anthrax, we can utilize the *B. anthracis* model to better understand how this bacterium adheres to and becomes internalized by epithelial cells, since the mechanisms that mediate these processes are independent of each other and not fully defined (24, 25). Furthermore, understanding of these processes can be used in the rational design of drugs that can mimic the effects conferred by rifaximin for the treatment of infections caused by this or similar pathogens.

The lack of effect of rifaximin pretreatment on *S. sonnei* adherence and internalization does contradict a recent study that established the use of rifaximin for the treatment of shigellosis (27), since the data presented in the present report examine only the effect of rifaximin on epithelial cells and do not address its antibiotic properties or its use in the treatment of shigellosis.

Since *B. anthracis* and *S. sonnei* utilize vastly different mechanisms of adherence and internalization (zipper versus trigger, respectively) (3, 24, 25), we can speculate as to the potential cellular compartments affected by rifaximin, since one bacte-

rium was affected and the other was not. Specifically, *S. sonnei* utilizes a sophisticated type III secretory system involving various host cell-signaling molecules that affect the cytoskeleton. *B. anthracis* internalization also involves various host cell secondary messengers and modifications to the cytoskeleton for internalization; however, various differences exist. For example, *B. anthracis* internalization does not require the membrane-cytoskeletal protein vinculin for internalization (Y. Xu, unpublished data), but this component does play a critical role in *Shigella* internalization by anchoring adhesion complexes to the actin cytoskeleton (3).

Since rifaximin is related to ansamycins (e.g., rifampin, rifabutin, rifalazil), which have been reported to have anti-inflammatory and immunosuppressive properties, and because several studies have suggested that rifaximin had effects on mammalian cell function, we chose to examine the cytokine profile in the supernatants of rifaximin-treated (and control) HEp-2 cells (16, 17). Defining the cytokine production profile was a rapid means of detecting potential changes in host cell physiology, in addition to characterizing this response in epithelial cells. This analysis is different from those of previous studies examining the effects of ansamycins on immune cell function and cytokine production. Specific to rifaximin, however, is a previous finding that humans with uncomplicated diverticular disease treated with rifaximin showed altered expression patterns of adhesion molecules associated with T-cell homing to the mucosa (2). A second study examined the role of rifaximin, alone or in the presence of a rifaximin-resistant probiotic *Bifidobacterium infantis* strain, on cytokine production by peripheral blood mononuclear cells (PBMCs) or the intestinal enterocytic HT29 cell line. This study by Vitali et al. demonstrated that rifaximin had the capacity alone (or in the presence of *B. infantis*) to increase PBMC production of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  and that the production of IL-8 by HT29 cells was increased if rifaximin was administered alone (28). IL-8 production by HT29 cells was significantly reduced if rifaximin was administered with *B. infantis* (28). The last observation is of interest because IL-8 has been shown to play a role in the severity of EAEC infections (11), suggesting that IL-8 downregulation may ameliorate disease-related symptoms; however, the Vitali study showed that rifaximin alone increases IL-8 production, and in the present report, rifaximin downregulated IL-8 (and most cytokines detected in the supernatants of untreated and control antibiotic-treated HEp-2 cells). The two primary differences between our study and that of Vitali et al. were the cell type used (HEp-2 versus HT29 cells) and the dose of rifaximin (64 versus 100  $\mu$ g). Furthermore, a caveat to our study is that we were not able to assess the effect of rifaximin plus EAEC on cytokine production or to determine the effects on the adherence of bacteria added to HEp-2 cells first and then treated with rifaximin, since we do not at this time have a rifaximin-resistant EAEC isolate with which to carry out these experiments.

Although we report here that rifaximin pretreatment significantly affected the natural cytokine expression profile of HEp-2 cells, we cannot from these data determine whether the downregulation of cytokines (or the upregulation of a yet undefined inhibitory factor) following rifaximin treatment was responsible for the reduced adherence observed. The mechanism(s) resulting in altered EAEC adherence likely involved



direct rifaximin-mediated alterations to the HEP-2 cells, which in turn affected the bacterial adherence pattern (directly, indirectly, or both). Furthermore, the observation that the effects were time and concentration dependent suggested that a rifaximin-mediated process was altering cellular parameters important to EAEC adherence. The likelihood that the presence or absence of a factor in the supernatant directly affected bacterial attachment was observed when bacteria were mixed with dialyzed supernatants obtained from HEP-2 cells treated with different antibiotics. Only dialyzed supernatants from rifaximin-treated cells altered bacterial attachment to HEP-2 cells not previously treated with rifaximin. Adherence was also reduced in the absence of HEP-2 cells, i.e., with glass only, suggesting that factors (or a lack thereof) in the supernatant were interacting with EAEC directly, thereby changing the adherence potential of the bacteria.

The data presented in this report suggested that rifaximin-mediated changes to various epithelial cell types reduced EAEC attachment by biologically altering the host cell. These effects extend to *B. anthracis* adherence and internalization but not to *S. sonnei*, suggesting that rifaximin-mediated changes in cell physiology did not have an effect on all bacteria. These observations suggested that significant functional differences between rifaximin and its cousin rifampin exist. Experiments designed to identify the factor(s) associated with rifaximin alteration of epithelial cell binding are currently under way.

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