Enteric dysbiosis promotes antibiotic-resistant bacterial infection: systemic dissemination of resistant and commensal bacteria through epithelial transcytosis

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Am J Physiol Gastrointest Liver Physiol 307: G824–G835, 2014. First published July 24, 2014; doi:10.1152/ajpgi.00070.2014.—Antibiotic usage promotes intestinal colonization of antibiotic-resistant bacteria. However, whether resistant bacteria gain dominance in enteric microflora or disseminate to extraintestinal viscera remains unclear. Our aim was to investigate temporal diversity changes in microbiota and transepithelial routes of bacterial translocation after antibiotic-resistant enterobacterial colonization. Mice drinking water with or without antibiotics were intragastrically gavaged with ampicillin-resistant (Amp-r) nonpathogenic Escherichia coli (E. coli) and given normal water afterward. The composition and spatial distribution of intestinal bacteria were evaluated using 16S rDNA sequencing and fluorescence in situ hybridization. Bacterial endocytosis in epithelial cells was examined using gentamicin resistance assay and transmission electron microscopy. Paracellular permeability was assessed by tight junctional immunostaining and measured by tissue conductance and luminal-to-serosal dextran fluxes. Our results showed that antibiotic treatment enabled intestinal colonization and transient dominance of orally acquired Amp-r E. coli in mice. The colonized Amp-r E. coli peaked on day 3 postinoculation and was competed out after 1 wk, as evidenced by the recovery of commensals, such as Escherichia, Bacteroides, Lachnospiraceae, Clostridium, and Lactobacillus. Mucosal penetration and extraintestinal dissemination of exogenous and endogenous enterobacteria were correlated with abnormal epithelial transcytosis but uncoupled with paracellular tight junctional damage. In conclusion, antibiotic-induced enteric dysbiosis predisposes to exogenous infection and causes systemic dissemination of both antibiotic-resistant and commensal enterobacteria through transcytotic routes across epithelial layers. These results may help explain the susceptibility to sepsis in antibiotic-resistant enteric bacterial infection.

gut-derived sepsis; superbug; intestinal microbiota; epithelial barrier; bacterial endodysbiosis

HIGH INCIDENCE OF BLOODSTREAM INFECTION with antibiotic-resistant bacteria or superbug has been reported in critical care units, of which the clinical isolates are mostly members of the Enterobacteriaceae family (e.g., Escherichia coli (E. coli) and Klebsiella pneumoniae) (29, 30, 34). Bacteria of the Enterobacteriaceae family are abundant in human feces and are part of the gut commensal microbiota (13, 33). It is generally believed that intestinal colonization of antibiotic-resistant bacteria may increase the risk of sepsis by selective overgrowth and dissemination in individuals who are clinically compromised.

One of the risk factors of antibiotic-resistant bacterial infection is previous history of antibiotic usage (34). Antibiotic treatment alters the intestinal microbiota, causing enteric dysbiosis (quantitative, qualitative, metabolic, or locational imbalances of gut commensals), which can take weeks to recover from after antibiotic withdrawal (16, 41). Previous studies in animal models showed that oral administration of antibiotics increase the susceptibility to pathogenic infection (9, 15, 26). Recent studies have indicated that usage of antibiotic cocktails induces clonal expansion and promotes colonization of multidrug-resistant enteric bacteria (1, 21, 35, 47), suggesting dominance of resistant organisms over commensals under antibiotic pressure. However, the time frame of antibiotic-resistant bacterial colonization and its relationship with commensal recovery after antibiotic withdrawal remain unclear.

Intestinal epithelial cells linked by tight junctions normally form a physical barrier to confine luminal bacteria (52). Once the barrier is breached, enteric bacteria may gain access to lamina propria through either transcellular or paracellular routes, causing microbial translocation to extraintestinal organs and systemic inflammation (17, 50). Abnormal adherence and internalization of commensal bacteria in epithelial cells have been documented in patients and experimental models with infection of Campylobacter jejuni (19) and Giardia lamblia (4), Crohn’s disease (22, 46), celiac disease (12, 43), chronic psychological stress (14), surgical manipulation (11), and intestinal obstruction (39). However, the underlying mechanism of enterobacterial dissemination in antibiotic-induced dysbiosis is largely unknown. The double impact of commensal dysbiosis and exposure to high numbers of resistant bacteria on intestinal barrier functions has also yet to be explored.

Our specific aims were to investigate the colonization time course of exogenously administered resistant enterobacteria in normal flora and under antibiotic-induced dysbiosis, temporal diversity changes in the intestinal microbiota following exogenous bacteria colonization, and the mechanisms of enterobacterial dissemination to the mucosa and extraintestinal organs.
MATERIALS AND METHODS

Animals. Specific pathogen-free BALB/c and C57BL/6 mice (5–7 wk of age, male) obtained from the Animal Center of the National Taiwan University were used. Myosin light-chain kinase-knockout (MLCK-KO) mice (B6 background) lacking the long 210-kDa MLCK were kind gifts from Dr. J. R. Turner, University of Chicago (8). Animals were raised in a temperature-controlled room (20 ± 2°C) with 12-h:12-h light/dark cycles and fed with regular mouse chow and water. All experimental procedures were approved by the Animal Care and Use Committee of the National Taiwan University.

Antibiotic-resistant bacterial strains. E. coli BL21 were transformed with pRSET containing an ampicillin-resistant (Amp-r) gene (a gift from Dr. Meng-Chun Hu, Graduate Institute of Physiology, National Taiwan University College of Medicine). E. coli BL21, a nonpathogenic laboratory strain derived from normal commensals of human gut, was originally isolated from human feces (10). Transformed E. coli were grown to log phase in Luria-Bertani (LB; Sigma, St. Louis, MO) broth containing 37°C 0.1 mg/ml ampicillin with vigorous shaking at 37°C. Colony-forming units (CFU) per milliliter were determined by spectrophotometry, and the required concentration of Amp-r E. coli was prepared in phosphate-buffered saline (PBS) for oral gavage.

Experimental design. Mice were drinking normal water (NW) or antibiotic water (AW) containing mixtures of vancomycin (500 mg/l), neomycin (1 g/l), metronidazole (1 g/l), and ampicillin (1 g/l) (36) for 1 wk before oral gavage with Amp-r E. coli. Antibiotics were all purchased from Sigma. Mice were inoculated with 10⁶ CFU of Amp-r E. coli in 0.2 ml PBS on day 0 and were given normal water for drinking afterward. On day 0 (before infection) and postinoculation (PI) days 1, 3, 7, and 14, mice were killed for sample collection.

Quantification of total and Amp-r bacteria in intestines. To evaluate gut-associated bacterial colonization, 1-cm segments of midjejunum, cecum, and colon were excised and cut longitudinally, and the luminal contents were rinsed off. Tissues were weighed aseptically and transferred to sterile PBS at a ratio of 1 mg to 10 μl for homogenization and sonication. The lysate was plated on LB agar plates with or without 50 μg/ml ampicillin for aerobic and anaerobic bacterial culture overnight at 37°C, and the total and Amp-r bacterial counts were normalized per gram of intestinal tissues (CFU/g), representing the colonized bacterial numbers.

The bacterial numbers in jejunal chyme as well as in fecal samples of the cecum and colon were also examined. The luminal contents of each intestinal segment were collected and weighed, followed by homogenization and sonication in sterile PBS at a ratio of 1 mg to 10 μl. Diluted samples were plated onto LB agar plates with or without 50 μg/ml ampicillin for aerobic and anaerobic culturing overnight, and the total and Amp-r bacterial CFUs were normalized per gram of luminal content (CFU/g), representing luminal bacterial numbers.

Analysis of bacterial translocation to extraintestinal organs. The livers and spleens were removed from animals with sterile instruments, and tissue weights were determined. The tissues were homogenized and sonicated in sterile PBS at a ratio of 1 mg to 10 μl. Undiluted lysates were plated onto LB agar plates with or without 50 μg/ml ampicillin for aerobic bacterial culturing. Following incubation at 37°C for 24 h, bacterial colonies were counted and normalized to CFU/g (28, 51).

Quantification of epithelial endocytosed bacteria. Segments of midjejunum (10 cm) and colon (2 cm) were excised, and the luminal contents were rinsed out with sterile PBS as previously described (4, 51). The tissues were placed inside-out on a steel rod and incubated in PBS containing 0.5 mM dithiothreitol (Sigma) for 10 min at room temperature to remove mucus. The segments were transferred into PBS with 30 mM ethylenediaminetetraacetic acid (EDTA) and 5.55 mM glucose and incubated for 20–30 min at 37°C and shaken gently. The solution was then passed through a nylon mesh with a pore size of 40 μm (BD Bioscience, San Jose, CA) to retain epithelial sheets.

The epithelial sheets were washed out from the inverted mesh with PBS and separated into single cells by gentle pipetting. The viability and purity of enterocytes were determined in a pilot study, showing Trypan blue negativity (91.7 ± 0.6%), BerEP4 (an epithelial marker) positivity (91.1 ± 1.2%), and a lack of CD68 (a macrophage marker) transcripts in the cell preparation.

The cells (2 × 10⁶) were then incubated with 300 μg/ml gentamycin (Invitrogen, Carlsbad, CA) for 1 h with gentle shaking. After being washed twice with PBS, cells were incubated with 1% Triton X-100 in PBS for 10 min on ice. Our pilot study confirmed that none of the intestinal bacteria was resistant to gentamycin. The lysate (200 μl) was plated onto agar plates for aerobic bacterial culturing overnight at 37°C. The numbers of bacterial colonies are presented as CFU per 10⁶ cells (4, 51).

Temporal temperature gradient gel electrophoresis and 16S rDNA sequencing. Colon and cecal stool samples of the mice (0.1–0.5 g wet weight) were immediately diluted 10-fold in peptone/water containing 20% glycerol and stored at 20°C until analysis. DNA (in a 0.2-ml volume) was isolated from the diluted feces samples using the QIAamp DNA stool mini kit. Then, stool DNA was amplified by PCR using a TaqGold Thermocycler (Biometra, Göttingen, Germany) with universal bacterial primers for 16S rDNA (314F with 40 bp GC at the 5′ end and 534R of E. coli) (2, 4). The PCR products were separated by temporal temperature gradient gel electrophoresis (TTGE) in a 10% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide) and 7 M urea (J.T. Baker, Phillipsburg, NJ). Premigration was conducted at 20 V and 55°C over a 15-min period. Gels were run for 6 h at 150 V with the temperature increasing at 2.5°C/min from 55 to 70°C. The DNA fragments were visualized using SYBR Green staining (Life Technologies, Carlsbad, CA), and the gel was scanned using a Gel System MultiGel-21 (Tobopbio, Taipei, Taiwan).

TTGE bands were excised from the gel and reamplified with the same 341F and 534R primers but without the GC clamp. The PCR products were digested and subcloned into the pGEM-T Easy Vector Systems (Promega, Madison, WI) and transformed into cells. The TTGE fragment insert was then sequenced (Applied Biosystems, Foster City, CA) and classified using the Classifier Tool provided by the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/classifier/classifier.jsp). The number of bands, indicative of bacterial diversity, was determined.

Fluorescence in situ hybridization. Microbiological analysis in intestinal tissues was conducted as described previously (4, 51). Briefly, intestinal segments (1 cm) were excised and cut longitudinally, and the luminal contents were rinsed off. Tissues were fixed in Carnoy’s solution (Ricca Chemical, Arlington, TX), embedded in paraffin, and sectioned at 5 μm. The sections were de waxed, placed in 1% Triton X-100, and washed in PBS three times before incubation in 5 mg/ml lysozyme at 37°C for 20 min. After PBS washing, the sections were incubated in prewarmed hybridization solution containing 0.02–0.1 μM oligonucleotide probes targeting bacterial 16S rDNA (see below) at 46°C overnight. The sections were then rinsed in washing buffer and air-dried before being stained with a Hoechst dye. The slides were mounted and viewed under a fluorescent microscope (Axio Imager A1; Carl Zeiss, Jena, Germany) equipped with a CCD camera and imaging software.

The probes included 5′-end FITC-labeled universal bacterial probe (EUB338) (5′-GCTGCTCCTCCGGTAGGACT-3′) and negative control probe (EUB138) (5′-ACATCTACGGGACGC-3′), as well as 5′-end Cy3-labeled probe for Lactobacillus/Enterococcus (Labi58) (5′-GGTATTAGCACCCTTGTTTCA-3′), E. coli (Ecol1513) (5′-ACCCTAGTGGCCTGTCATA-3′), and Bacteroides (Bac 303) (5′-CCAATTGGGGACCTT-3′) (Genomics BioScience and Technology, Taipei, Taiwan).

Transmission electron microscopy. Intestinal tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 22°C and rinsed with 0.05 M Tris buffer (pH 7.4) for 4 h at 4°C. Tissues were osmicated, dehydrated in a graded ethanol
series, and embedded in epoxy resin. Thick vibratome sections (100 μm) were cut and washed in Tris buffer three times for 5 min each. Thin sections (70 nm) were then cut and examined in a Hitachi-7100 electronmicroscope equipped with a digital system. Electronmicrographs of epithelial cells were taken at magnifications of ×6,000 and ×15,000 (50).

**Histological examination.** Intestinal segments were fixed in 4% paraformaldehyde (PFA), and care was taken to ensure proper orientation of the crypt-to-villus axis during embedding. Sections of 4-μm thickness were stained with hematoxylin and eosin and observed under a light microscope.

**Ussing chamber studies and macromolecular flux assay.** Intestinal segments were opened along the mesenteric border and mounted in Ussing chambers (WPI Instruments, Worcester, MA) as previously described (17, 50). The opening of the chamber (1 cm²) exposed the tissue to 5-ml circulating oxygenated Krebs buffer. The serosal buffer contained 10 mM NaCl-glucose that was osmotically balanced with 10 mMol/l mannitol in the mucosal buffer. A circulating water bath maintained the temperature of the buffer at 37°C. The potential difference (PD) between the two compartments was clamped at 0 V using a voltage clamp amplifier, and the short-circuit current (ISC) of the tissue was then determined. The tissue conductance (G) was calculated according to Ohm’s law by dividing the value of ISC by that of PD.

Intestinal permeability was determined by the level of mucosal-to-serosal flux of dextran conjugated to FITC (dextran-FITC, molecular weight = 4 kDa; Sigma). Tissues mounted on the Ussing chambers were allowed to equilibrate until the ISC stabilized before dextran probe was added to the mucosal buffer at a final concentration of 500 μM. Samples (250 μl) of serosal buffer were collected at 0, 30, 60, and 90 min after addition of dextran probe and were replaced with Krebs buffer/glucose. The fluorescence units of dextran-FITC in serosal buffer were determined at excitation/emission 490/530 nm using a multi-mode plate reader (Beckman Coulter, Fullerton, CA), and the concentration (nM) was calculated according to a standard curve.

**Electron microscopy.** Intestinal tissue was fixed with 4% PFA for 1 h at 4 °C and then snap-frozen in optimal cutting temperature solution (Thermo Fisher Scientific, San Francisco, CA) overnight at 4°C. After being washed with PBS, tissues were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1,000; Molecular Probes, Eugene, OR) and further incubated with goat anti-mouse IgG or anti-rabbit IgG (1:1,000, Cell Signaling Technology). Tissues mounted on the Ussing chambers (WPI Instruments, Worcester, MA) as previously described (17, 50). The opening of the chamber (1 cm²) exposed the tissue to 5-ml circulating oxygenated Krebs buffer. The serosal buffer contained 10 mM NaCl-glucose that was osmotically balanced with 10 mMol/l mannitol in the mucosal buffer. A circulating water bath maintained the temperature of the buffer at 37°C. The potential difference (PD) between the two compartments was clamped at 0 V using a voltage clamp amplifier, and the short-circuit current (ISC) of the tissue was then determined. The tissue conductance (G) was calculated according to Ohm’s law by dividing the value of ISC by that of PD.

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**Results.** Antibiotic treatment decreases intestinal bacterial numbers in mice. To verify absence of resistant strains in intestines and antibiotic killing of commensals, the gut-associated and luminal bacterial counts were determined by culturing tissue lysates and luminal contents, respectively, after 1 wk of NW and AW treatment. A two- to four-log decrease in gut-associated bacterial counts was seen in the jejunum, cecum, and colon of AW mice, compared with NW controls (Table 1). Luminal bacterial

<table>
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<th>AW</th>
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<tr>
<td>Cecum</td>
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Table 1. Gut-associated and luminal bacterial counts in mice drinking NW and AW for 1 wk, and in those after withdrawal of AW for 14 days

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Values represent means ± SE. The tissue homogenate and luminal content of each intestinal segment were plated on agar plates under aerobic and anaerobic conditions. NW, normal water; AW, antibiotic water; ND, not detected. CFU, colony-forming units. *P < 0.05 vs. NW. n = 8–10/group.
numbers were also significantly lower in AW mice than NW mice (Table 1). After antibiotic removal for 14 days, the bacterial counts in AW mice returned to normal levels, comparable to those drinking NW (Table 1). Moreover, no bacterial colony was found on ampicillin-containing agar plates, suggesting that antibiotic-resistant bacteria were not present in the intestines of either group.

Intestinal colonization of Amp-r bacteria is seen only in mice after antibiotic treatment. In the following experiments, mice were orogastrically inoculated with Amp-r E. coli to evaluate exogenous colonization of resistant bacteria. The total and Amp-r bacterial counts in jejunal, cecal, and colonic tissues were determined on day 0 (before inoculation), or PI days 1, 3, 7, and 14. In NW mice, none of the intestinal segments showed presence of Amp-r bacteria at any time point (Fig. 1A). The total bacterial numbers remained constant in cecal and colonic segments after inoculation of Amp-r E. coli; a decrease without statistical significance was seen in jejunal counts on PI day 3 (Fig. 1A). The anaerobic bacterial counts showed a similar trend to those of aerobic culturing, with no sign of Amp-r E. coli colonization in NW mouse intestines (data not shown).

In contrast, increased numbers of Amp-r bacteria were seen colonizing the cecal and colonic segments of AW mice compared with NW mice on PI day 3 (Fig. 1B). The numbers of colonized-resistant strains were higher in the cecum and colon than in the jejunum on PI day 3, whereas no Amp-r bacteria were detected after 7 days in any segment (Fig. 1B). The Amp-r bacteria were identified to be Escherichia genus by 16S rDNA sequencing. The total bacterial counts in intestinal tissues increased two- to four-log on PI days 1–3 and plateaued afterward, compared with day 0, in AW mice (Fig. 1B). Additionally, the luminal numbers of Amp-r bacteria in fecal samples of colon and cecum also peaked on PI day 3 in AW mice, whereas none was found in jejunal luminal contents (data not shown).

Clearance of Amp-r bacteria is associated with recovery of commensal microbiota. The diversity of enteric microbiota was examined using TTGE and 16S rDNA sequencing, whereby DNA extracted from colonic stool was amplified using universal bacterial primers and separated into multiple bands on gradient gels. The band numbers amplified from colonic samples of NW mice were similar on day 0 (before inoculation) and 1, 3, 7, and 14 days after oral gavage of Amp-r E. coli (Fig. 2, A and B), indicating no alteration in bacterial diversity. Several bacterial taxa were identified in the NW colonic stool, such as Bacteroides, Porphyromonadaceae, Escherichia, Mucispirillum, Lactobacillus, Lachnospiraceae, Clostridium, and Ruminococcaceae.

Decreased band numbers were found in the colonic stool of AW mice compared with NW mice on day 0, indicating a loss of microbial diversity after antibiotic treatment (Fig. 2, A–C). The degree of bacterial diversity increased gradually and was close to normal levels by PI day 14 in AW mice (Fig. 2, A and C), suggesting recovery of commensal microbiota in about 2 wk after antibiotic withdrawal. The recovered bacterial strains on PI day 14 in AW mice included Escherichia, Bacteroides, Lactobacillus, Lachnospiraceae, and Clostridium.

Mucosal penetration and extraintestinal translocation of both resistant and commensal bacteria in antibiotic-treated mice. Normal morphological and histological structures of gastrointestinal tracts were seen in NW mice before and after inoculation of Amp-r E. coli and also in AW mice on PI day 0 (before inoculation) (data not shown), suggesting that antibiotics treatment per se did not cause structural damages. Cecal flatulence or distension associated with tissue hyperemia and edema were evident in AW mice on PI days 1 and 3, of which

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**Fig. 1.** Total and ampicillin-resistant (Amp-r) bacterial (bac) counts in mouse intestines by aerobic culturing. Intestinal homogenates of normal water (NW) (A) and antibiotic water (AW) (B) mice postinoculated with Amp-r Escherichia coli (E. coli) were aerobically cultured on agar plates with or without ampicillin. Data are expressed as means ± SE. The dashed line indicates that no colony was detected on agar plates. *P < 0.05 vs. NW; #P < 0.05 vs. day 0, n = 5–6/group. The experiments were repeated twice.
the structures returned to normal on PI days 7–14 (data not shown). The jejunal and colonic histology in AW mice remains intact after colonization of resistant bacteria (data not shown).

We next evaluated the presence of bacteria in gut mucosa by in situ hybridization. To avoid contamination with luminal bacteria, chyme or fecal contents were washed off before tissue processing. No sign of bacteria was seen in intestinal mucosa (Fig. 3A) and extraintestinal organs (liver and spleen; Fig. 3D) of NW mice after inoculation of Amp-r E. coli. In contrast, bacteria were found in the intestinal crypts, lamina propria, and epithelial layer of AW mice (Fig. 3B). By hybridization with probes targeting specific bacterial strains, penetration of E. coli, Bacteroides, and Lactobacillus was noted in the gut mucosa of AW mice (Fig. 3C). Moreover, augmentation of bacterial translocation to the liver and spleen was seen on PI day 3 in AW mice compared with NW mice (Fig. 3, D and E).

**Presence of transcellular but not paracellular epithelial barrier dysfunction.** To investigate the routes for enteric bacterial translocation, transcellular and paracellular epithelial permeability was assessed. First, intracellular bacterial counts were measured in purified epithelial cells isolated from jejunal, cecal, and colonic segments using a gentamycin assay. The epithelial endocytosed bacterial count was minimal in NW mice (Fig. 4A), whereas a significant increase in bacterial numbers was found in the small and large intestinal epithelial cells in AW mice during peak colonization on PI day 3 (Fig. 4B). The presence of Amp-r bacteria was noted in the enterocytes of the cecum and colon of AW mice (Fig. 4B), of which the resistant bacteria were identified to be Escherichia genus by 16S rDNA sequencing. Moreover, electrophoretic images revealed bacterial internalization to jejunal and colonic epithelial cells in AW mice on PI day 3 after inoculation, whereas no sign of intracellular bacteria was seen in NW mice (Fig. 4C).

The tissue conductance, luminal-to-serosal dextran flux, and tight junctional structures were next examined to verify changes in paracellular permeability. In both NW and AW mice, inoculation of Amp-r E. coli (PI day 3) did not alter the tissue conductance or dextran flux in jejunal and colonic segments, compared with day 0 (Fig. 5, A and B). Furthermore, normal honeycomb pattern of the ZO-1 and full-length occludin without cleavage were seen in the intestinal mucosa of both NW and AW mice (Fig. 5, C and D). Intact tight junctions were also observed in electronmicrographs of intestinal epithelial cells in both NW and AW mice on PI days 0 and 3 (Fig. 4C). Taken together, the results suggested that neither antibiotic treatment nor bacteria inoculation caused tight junctional damage.

Additional experiments were conducted to elucidate the mechanisms of transcellular passage of enteric bacteria. Our previous study had shown that long MLCK-210 kDa was involved in bacterial endocytosis by small intestinal epithelial cells in a mouse model of bowel obstruction (51). The activation of MLCK-210 caused terminal web myosin phosphorylation and brush border fanning, allowing bacterial penetration through intermicrovilli clefts in enterocytes. To assess the role of MLCK-210 in the mechanism of bacterial endocytosis following antibiotic-resistant bacterial colonization, wild-type (WT) mice and MLCK-210-KO mice were orally inoculated with Amp-r E. coli after they drank NW or AW. Increased numbers of gut-associated total and Amp-r bacterial counts were found in the large intestines of both mouse strains drinking AW compared with NW, suggesting comparable levels of antibiotic-resistant bacterial colonization in the two mouse strains (Fig. 6, A and B). In parallel with Amp-r bacterial colonization, the epithelial endocytosed bacterial counts in the large intestines of both mouse strains were also higher in groups drinking AW compared with NW (Fig. 6, C and D). The intracellular total bacterial counts in intestinal epithelial cells of MLCK-KO mice were slightly lower than WT mice (64 ± 23 vs. 287 ± 136 CFU/10⁶ cells, P > 0.05) (Fig. 6C), albeit without statistical significance. An
insignificant decrease of the numbers of endocytosed Amp-r bacteria was found in MLCK-KO mice compared with those of WT mice (12 ± 3 vs. 33 ± 13 CFU/10⁶ cells, P > 0.05) (Fig. 6D).

Increase in proinflammatory signals and cytokines in gut tissues. Lastly, the phosphorylation levels of proinflammatory signaling pathways (JNK, ERK, p38, IκBα) were examined in gut mucosa following antibiotic-resistant bacterial coloniza-

Fig. 3. Bacterial penetration into gut mucosa and extraintestinal organs of AW mice after infection with Amp-r E. coli. A and B: jejunal, cecal, and colonic segments of NW and AW mice were subjected to fluorescence in situ hybridization with probes targeted to universal bacteria (green) and E. coli (red). Cell nuclei (blue) have been superimposed on the images. Representative photomicrographs (magnification ×200) were obtained from at least 5 mice per group. Inset: higher magnification of bacteria. C: penetration of E. coli, Bacteroides, and Lactobacillus to intestinal mucosa of AW mice on PI day 3. D and E: extraintestinal bacterial counts in liver and spleen homogenates of NW and AW mice. *P < 0.05 vs. NW; #P < 0.05 vs. day 0. n = 5/group. CFU, colony-forming units.
Fig. 4. Increased bacterial endocytosis in intestinal epithelial cells of AW mice after inoculation of Amp-\( r \) E. coli. The number of intracellular bacteria was determined in jejunal and colonic epithelial cells of NW mice (A) and AW mice (B), using a gentamycin resistance assay. \(*P < 0.05\) vs. NW; \(#P < 0.05\) vs. day 0. \( n = 5\) group. The experiments were repeated at least twice. C: electromicrographs showing bacterial internalization to epithelial cells in AW mice. Representative images showing jejunal and colonic epithelial cells of NW and AW mice on PI day 0 (before infection) and day 3 after orogavage of antibiotic-resistant bacteria. Intracellular presence of bacteria (arrowheads) was seen in jejunal and colonic segments of AW mice after infection but not in NW mice. Intact tight junctions (TJ) were seen in epithelial cells in all groups. Scale bar = 1 \( \mu \)m. \( n = 5\) group.
Comparable levels were seen before (day 0) and after (day 3) inoculation of Amp-r E. coli in mice drinking NW (data not shown). On the other hand, upregulated phosphorylation of IκBα and ERK in colonic mucosa was seen after Amp-r E. coli colonization in mice drinking AW (Fig. 7, A and B). No difference in phosphorylated levels of JNK and p38 was seen after resistant bacterial colonization in AW mice (Fig. 7, C and D). Moreover, increased IFN-γ levels in gut tissues were noted after infection in AW mice compared with NW mice (Fig. 7E). The TNF-α level was not different among groups (Fig. 7F).
DISCUSSION

Emergence of antibiotic-resistant bacteria has become a worldwide public health problem, challenging clinical microbiologists and infection control professionals. Previous studies showed that antibiotic usage promotes colonization and selective expansion of antibiotic-resistant enterobacterial species in the intestinal tract (1, 21, 35, 47). Novel findings from our study indicate that transient dominance of exogenous enterobacteria caused by antibiotic manipulation may lead to microbial dissemination to extraintestinal viscera through transcytotic passage. We demonstrated that transcellular microbial entry was uncoupled with tight junctional damage, and the invasiveness was limited to antibiotic-resistant strains and also to commensals.

Orogastric gavage of antibiotic-resistant 


$E.\ coli$


did not lead to intestinal colonization in mice with normal flora. It is noteworthy that a decrease, albeit with statistical insignificance, of total bacterial counts was seen in the NW mouse jejunum 3 days after inoculation. We speculate that orogastric gavage of Amp-r 


$E.\ coli$


colonization. Wild-type (WT) mice and MLCK- knockout (KO) mice were orally inoculated with Amp-r 


$E.\ coli$


after drinking NW or AW, and large intestinal tissues were collected for analysis. A and B: numbers of gut-associated total and Amp-r bacteria colonized in large intestines were determined by culturing tissue homogenates. C and D: intracellular total and Amp-r bacterial counts in epithelial cells were also determined. *$P < 0.05$ vs. NW. n = 6–8/group.

Fig. 6. Role of myosin light-chain kinase (MLCK)-210 in mechanisms of bacterial endocytosis by intestinal epithelial cells after Amp-r 


$E.\ coli$


colonization. Wild-type (WT) mice and MLCK- knockout (KO) mice were orally inoculated with Amp-r 


$E.\ coli$


after drinking NW or AW, and large intestinal tissues were collected for analysis. A and B: numbers of gut-associated total and Amp-r bacteria colonized in large intestines were determined by culturing tissue homogenates. C and D: intracellular total and Amp-r bacterial counts in epithelial cells were also determined. *$P < 0.05$ vs. NW. n = 6–8/group.
resistant bacterial infection. Abnormal epithelial endocytosis of commensals or nonpathogenic bacteria has been reported in animal models of infection with *Campylobacter jejuni* (19) and *Giardia lamblia* (4), psychological stress (44, 53), and chemically induced colitis (32). Moreover, presence of mucosal adherent and invasive bacteria or *E. coli* was documented in patients with inflammatory bowel disease, surgical stress, and necrotizing enterocolitis (3, 23, 37). In vitro epithelial cell cultures studies also showed the internalization and endocytosis of nonpathogenic bacteria under inflammatory conditions and metabolic stress, such as low-dose IFN-γ (6), TNF-α during glutamine deprivation (7), uncoupling of mitochondrial oxidative phosphorylation (27), and hypoxia (49). Although we cannot rule out the possibility of cells with phagocytic functionality (e.g., M cells and dendritic cells) for internalizing bacteria (45), our in situ hybridization data did show augmented adherence and colocalization of bacteria with the large surface area of epithelial layers.

Our previous study had shown that long MLCK-210 kDa was involved in terminal web myosin phosphorylation and brush border fanning, allowing bacterial penetration through intermicrovillus clefts in small intestinal epithelial cells, in a mouse model of bowel obstruction (51). In the current model of antibiotic-resistant bacterial colonization, the lack of MLCK-210 appears to decrease the level of microbial internalization by large intestinal epithelial cells, albeit without statistical significance. We speculate that the dependency on MLCK-210 for bacterial endocytosis may differ between epithelial cells with short vs. long brush border. Because antibiotic-resistant bacteria colonize mainly the large intestine rather than the small intestine, alternative mechanisms other than MLCK-210 may be responsible for bacterial internalization.

The enterobacterial penetration and dissemination paralleled the colonization time frame of Amp-r *E. coli*, both of which peaked on PI day 3 and were eliminated by day 14, indicating that recovery of commensal bacterial diversity, not only cleared out exogenous microbes, but also reestablished the epithelial defenses against luminal commensals. Several lines of evidence show that the normal microbiota is involved in the regulation of epithelial turnover and barrier fortification (24, 42). Previous studies have shown that administration of a single or mixtures of probiotics, such as *Lactobacillus spp.*, *E. coli Nissle 1917, Enterococcus faecalis*, and *Bifidobacterium brevis*, may prevent the epithelial permeability increase and bacterial translocation in various disease models (25, 31, 48, 53). Taken together, interventions fortifying the symbiont/probiotic population or epithelial barriers may be considered as adjunct therapy for patients receiving long-term antibiotic therapy.

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**Fig. 7.** Colonic inflammation was noted after Amp-r *E. coli* inoculation in mice drinking AW but not NW. Western blots showing the phosphorylation levels of IkB-α (A), ERK1/2 (B), p38 (C), and JNK (D) in colonic mucosa before (day 0) and after (day 3) inoculation of Amp-r *E. coli* in mice drinking AW. Band densities are expressed as means ± SE. Representative blots of at least 2 individual experiments. *P < 0.05 vs. day 0. n = 5–6/group. Levels of IFN-γ (E) and TNF-α (F) in colonic tissues of NW and AW mice were also evaluated. n = 5–6/group.
In conclusion, disruption of enteric microflora predisposes colonization of orally acquired antibiotic-resistant organisms, leading to systemic dissemination of both commensal and resistant bacteria through transcytotic routes in the absence of paracellular tight junctional damage. The subsequent gut-derived septic complications would be detrimental to immunocompromised individuals, who are often already on multiple long-term antibiotic regimens.

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REFERENCES


Author Contributions

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