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

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Renewed interest in antibiotic-resistant bacterial infection has been evinced by the recovery of commensals, such as Escherichia coli, 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 imbalance of gut commensals), which can take weeks to recover from after antibiotic withdrawal (16, 41). 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 bacterial 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 mice 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 BI21, a nonpathogenic laboratory strain derived from normal commensals of human gut, was originally isolated from human feces (10). A Amp-r E. coli were grown to log phase in LB (Sigma, St. Louis, MO) broth containing 370 μg/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 μg/ml), 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 10⁷ 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 (200 μl) were inoculated 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 mid-jejunum (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 gentamicin (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 gentamicin. 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. Colonic 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 T3000 Thermocycler (Biometra, Göttingen, Germany) with universal bacterial primers for 16S rDNA (341F 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 (Tobipho, 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 then rinsed in 51). The tissues were placed inside-out on a steel rod and incubated in 0.1 M sodium cacodylate buffer (pH 7.4), embedded in paraffin, and sectioned at 5 μm. The sections were then rinsed 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 viretome 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 mmol/l 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.

**Staining of tight junctional structures on intestinal tissues.** A 1-cm gut segment was fixed with 4% PFA for 1 h at 4°C and then snap-frozen in optimal cutting temperature solution (Thermo Fisher Scientific, Rockford, IL). Frozen tissues were cut into 6-μm sections with a cryostat and mounted on precoated glass slides for immunofluorescence staining following previously described protocols. Frozen tissue sections were prefixed in cold acetone for 10 min, air dried, and fixed again with 4% PFA for 2 min. The tissues were then permeabilized with 1% Triton X-100 for 1 min and blocked with fetal bovine serum for 1.5 h at room temperature. Tissue sections were then incubated with rabbit anti-zonula occludens (ZO)-1 (1:50; Zymed, 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 for 1 h at room temperature. Tissues were then stained for F-actin by labeling with phalloidin conjugated to Alexa Fluor 633 (5 nmol/l) and the concentration (nM) was calculated according to a standard curve.

**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 after antibiotic killing of commensals. The gut-associated and luminal bacterial counts were seen in the jejunum, cecum, and colon of AW mice. The gut-associated and luminal bacterial counts were determined by culturing tissue lysates after antibiotic killing of commensals. The gut-associated and luminal bacterial counts were seen in the jejunum, cecum, and colon of AW mice. The gut-associated and luminal bacterial counts were determined by culturing tissue lysates after antibiotic killing of commensals. The gut-associated and luminal bacterial counts were seen in the jejunum, cecum, and colon of AW mice. The antibiotic treatment decreased 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>
<thead>
<tr>
<th>Bacterial count, log_{10}CFU/g</th>
<th>NW</th>
<th>AW</th>
<th>Withdrawal</th>
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<tbody>
<tr>
<td><strong>Gut-associated bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J jejunum</td>
<td>4.41 ± 0.48</td>
<td>1.60 ± 0.67</td>
<td>5.49</td>
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<tr>
<td>Cecum</td>
<td>6.42 ± 0.11</td>
<td>3.02 ± 0.61*</td>
<td>6.09</td>
</tr>
<tr>
<td>Colon</td>
<td>6.27 ± 0.19</td>
<td>2.24 ± 0.60*</td>
<td>6.33</td>
</tr>
<tr>
<td>Anaerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J jejunum</td>
<td>4.19 ± 0.45</td>
<td>1.33 ± 0.71</td>
<td>5.58</td>
</tr>
<tr>
<td>Cecum</td>
<td>6.54 ± 0.24</td>
<td>3.12 ± 0.80*</td>
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</tr>
<tr>
<td>Colon</td>
<td>6.30 ± 0.20</td>
<td>2.10 ± 0.60*</td>
<td>6.74</td>
</tr>
<tr>
<td><strong>Luminal bacteria</strong></td>
<td></td>
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</tr>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J jejunum</td>
<td>6.24 ± 5.71</td>
<td>3.26 ± 3.22*</td>
<td>6.81</td>
</tr>
<tr>
<td>Cecum</td>
<td>8.55 ± 8.06</td>
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<tr>
<td>Colon</td>
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<td>ND*</td>
<td>8.12</td>
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<tr>
<td>Anaerobic</td>
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<tr>
<td>J jejunum</td>
<td>6.06 ± 5.65</td>
<td>1.90 ± 1.90*</td>
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<tr>
<td>Cecum</td>
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</tr>
<tr>
<td>Colon</td>
<td>7.04 ± 6.23</td>
<td>ND*</td>
<td>6.92</td>
</tr>
</tbody>
</table>

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

![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.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00070.2014/fig-1)
tight junctional structures were next examined to verify
(Fig. 4). Whereas no sign of intracellular bacteria was seen in NW mice
epithelial cells in AW mice on PI ages revealed bacterial internalization to jejunal and colonic
by 16S rDNA sequencing. Moreover, electromicrographic im-
the resistant bacteria were identified to be
cytes of the cecum and colon of AW mice (Fig. 4). The presence of Amp-r bacteria was noted in the entero-
numbers was found in the small and large intestinal epithelial
epithelial endocytosed bacterial count was minimal in NW 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 occlu-
din 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 electromicrographs 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 dam-
age.
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 intermicrovillus 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/10^6 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/10^6 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, IκB-α) were examined in gut mucosa following antibiotic-resistant bacterial coloniza-
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: electronmicrographs 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 μm. 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 Iκ-Bα 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 may have an impact on the numbers of normal commensals, possibly by stimulation of antimicrobial peptides. However, neither microbiota population change nor exogenous infection was seen in NW mice. On the other hand, colonization of antibiotic-resistant bacteria was observed after commensals were depleted by antibiotics. This finding is in keeping with previous studies (40, 47), supporting clinical observations that antibiotic usage may increase susceptibility to secondary infection. We also demonstrated that clearance of exogenous bacteria from the intestinal tract paralleled the recovery of commensal diversity after antibiotic withdrawal. The findings here bring novel information for strategies of vaccine design employing mucosal colonization of constructed bacteria for antigen delivery (5, 20). Future studies to tease out which commensals are important in competitive elimination of exogenous colonization and their mode of action are warranted.

Our results showed that intestinal colonization of antibiotic-resistant bacteria upon dysbiosis triggered the translocation of resistant and commensal bacteria to gut mucosa and extraintestinal organs. A previous study showed that oral antibiotics induce the expansion of multi-drug resistant enterobacterial strains from the population of commensal microbiota, of which the resistant strain may invade gut mucosa after chemical-induced colitis (1). We provided evidence here that both exogenous resistant strains and endogenous enterobacteria may translocate across the epithelial layer, and chemical disruption of epithelial barrier is not a prerequisite for enteric microbial penetration. In our study, bacterial influx was accompanied by increased proinflammatory signals and upregulated IFN-γ levels in gut tissues. The phenomenon of bacterial translocation and the subsequent gut-derived septic complications would be detrimental to immunocompromised individuals, such as patients with acquired immunodeficiency virus infection and those receiving immunosuppressant therapy, who are often already on multiple long-term antibiotic regimens (38).

Translocation of resistant and commensal bacteria to extraintestinal organs was observed despite normal epithelial tight junctional structures in antibiotic-treated mouse intestines. The results indicated that antibiotics alone did not render any of the gut segments permeable. Thus colonization and exposure to large numbers of resistant enterobacteria may be the main cause triggering enhanced transepithelial passage of microbes. We found a transient increase in intracellular bacteria counts in jejunal, cecal, and colonic epithelial cells after antibiotic-
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 breve, 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.

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 Ik-Bα (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 immuno-compromised individuals, who are often already on multiple long-term antibiotic regimens.

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