

Assessment of the role of antibiotics and enterococcal virulence factors in a mouse model of extraintestinal translocation

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Objective: To study the relative contribution of antibiotics and bacterial virulence factors in the process of translocation of *Enterococcus faecalis* from the gut to extraintestinal organs.

Design: Prospective controlled animal study.

Setting: Animal experimental laboratory at a university medical center.

Subjects: Fifty-two female Balb/c mice.

Interventions: We developed a mouse model to study the translocation of *Enterococcus faecalis* from the intestinal tract. Balb/c mice received sterile drinking water or antibiotic combinations to deplete their indigenous intestinal microflora. The animals subsequently were fed genetically engineered enterococci expressing different combinations of the putative enterococcal virulence factors aggregation substance and binding substance. Animals were killed, and their livers, spleens, and mesenteric lymph nodes were aseptically removed and cultured along with fecal samples for enumeration of bacteria.

Measurements and Main Results: All animals were colonized with the test strains at $2-6 \times 10^9$ colony forming units/g of feces; in the antibiotic-treated animals, feces were free from anaerobes and *Enterobacteriaceae*. In animals fed the identical bacterial mutant, the colony counts in mesenteric lymph nodes were sig-

nificantly lower in mice not treated with antibiotics than in those treated with antibiotics ($p = .016$). Multigroup analysis of variance revealed no significant differences of the translocation frequencies for the different mutant strains; however, the differences were statistically significant for all groups receiving antibiotics vs. the group not receiving antibiotics ($p < .05-.01$). There was a trend (although not statistically significant) for a higher proportion of positive cultures from either spleen or liver in mice that had enterococci recovered from their mesenteric lymph nodes (28%) relative to those that did not have enterococci isolated from the lymph nodes (12%; rate ratio 2.39, $p = .30$ by logistic regression analysis).

Conclusions: Oral antibiotics can select for extraintestinal translocation of *Enterococcus faecalis*, and neither aggregation substance nor binding substance seems to be required for this process. The experiments encourage further exploration of host and microbial factors contributing to translocation and may provide a better understanding of the pathogenesis of enterococcal infections in patients in intensive care units. (Crit Care Med 2004; 32:467-471)

KEY WORDS: intensive care unit infection pathogenesis; mouse sepsis model; enterococcal infections; translocation; enterococcal virulence factors; antibiotics;

The importance of enterococci as nosocomial pathogens is increasing, especially in patients in intensive care units, in newborns, in transplant recipients, and in patients with hematologic malignancies (1, 2). Enterococci are the third most common nosocomial bacterial pathogens isolated from the bloodstream of hospitalized patients and the second most common nosocomial isolates (3), with in-

fection rates increasing over the last 10 yrs (4).

Many aspects of the pathogenesis of severe enterococcal infections are still unclear. However, epidemiologic and experimental work points to the importance of the intestinal tract, which serves as a reservoir for endogenous enterococcal infections in hospitalized patients. Intestinal colonization with vancomycin-resistant enterococci is a risk factor for bloodstream infections caused by vancomycin-resistant enterococci (5). Furthermore, the same isolates—as determined by pulsed-field gel electrophoresis—caused nosocomial bloodstream infections and also were recovered from the patients' intestinal tracts (6). In addition to colonization, antibiotic use is considered a risk factor for the development of enterococcal bloodstream infections, especially when the antibiotics are active against anaerobic intestinal bacteria (5).

The importance of specific virulence factors is not as well established for enterococci as for other bacterial species. Among potential factors, enterococcal aggregation substance (AS), which is a 137-kD surface protein, repeatedly has been linked to enhanced pathogenicity of *Enterococcus faecalis*. AS is expressed more frequently by enterococci causing bloodstream infections and by isolates from liver transplant patients than by enterococci isolated from healthy volunteers (7). Furthermore, several experimental studies have indicated that AS interacts with cultured intestinal epithelial cells and may contribute to mucosal invasion and possibly to translocation of enterococci from the gut (8-11). When complexed with binding substance (BS), AS mediates a pheromone-responsive clumping reaction in *E. faecalis*. The clumping substantially enhances the transfer of plasmids, thereby contribut-

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ing to the spread of antibiotic resistance (12). Together with BS, AS has been shown to play an important role in the pathogenesis of experimental enterococcal endocarditis (13).

The difficulty in treating enterococcal infections and the lack of understanding of the mechanisms involved in their pathogenesis prompted us to investigate the specific role of AS, BS, and antibiotics in a mouse model of translocation from the gastrointestinal tract.

MATERIALS AND METHODS

Bacteria. Four isogenic mutants of *E. faecalis* OG1SSp were used throughout the experiments (kindly provided by G. Dunny, Minneapolis, MN) (13). *E. faecalis* OG1SSp constitutively expresses the chromosomally encoded BS, the structure of which is thought to be a lipoteichoic acid. When harboring the plasmid pINY1801, the strain additionally expresses AS, which leads to spontaneous clumping in liquid broth culture (AS+, BS+). *E. faecalis* OG1SSp containing the shuttle vector pWM401 without the gene encoding AS served as control (AS-, BS+). To separate specific interactions of AS and the clumping reaction itself, *E. faecalis* INY3000, which is a Tn916 transposon mutant of OG1SSp deficient for BS, also was used. *E. faecalis* INY3000 containing the plasmid pINY1801 was therefore positive for AS and negative for BS (AS+, BS-), whereas the control containing only the pWM401 plasmid expressed neither AS nor BS (AS-, BS-). All strains were grown in tryptic soy broth (Becton Dickinson, Cockeysville, MD) containing chloramphenicol (10 mg/L), and the growth medium for *E. faecalis* INY3000 also contained tetracycline (10 mg/L). The appropriate phenotypes were confirmed from inocula and from isolates recovered from the animal experiments by observing the macroscopically visible spontaneous clumping reaction in OG1SSp (pINY1801); OG1SSp (pWM401) and INY3000 (pINY1801) clump only when grown together in broth culture, whereas INY3000 (pWM401) does not clump in co-culture with either OG1SSp (pWM401) or INY3000 (pINY1801) (13). Colony-forming units (cfu) of inocula and of specimens derived from the experiments were determined by thoroughly vortexing all samples, sonicating them for 20 secs, and immediately plating them in appropriate dilutions onto agar media.

Animal Model of Translocation. A total of 49 female 6- to 8-wk-old Balb/c mice (17–21 g; Harlan Sprague Dawley, Indianapolis, IN) were studied after we obtained consent from the institutional review board for animal experiments. The mice were handled in accord with National Institutes of Health guidelines and with barrier precautions (gloves, gowns, face masks). The animals were housed in microisolator cages, which were opened only un-

der laminar-airflow hoods. After initial culture of fecal pellets, the following antibiotics were added to sterile drinking water for 10 days: vancomycin, gentamicin, and cefoxitin at 1.0 g/L and metronidazole at 0.1 g/L. Throughout the experiment, the antibiotic mixtures were freshly prepared and replaced every other day. Every 2–4 days, fecal pellets were obtained, homogenized, and diluted in phosphate-buffered saline and cultured aerobically on MacConkey agar for Gram-negative rods (Becton Dickinson) and on selective plates for enterococci (Enterococcosel, Becton Dickinson). The mice were placed into new cages every other day to prevent recolonization with intestinal bacteria by coprophagia. After the decolonization period, only cefoxitin (0.125 g/L) and metronidazole (0.1 g/L) were present in the drinking water, and 5×10^7 cfu/mL of one of the four mutants of *E. faecalis* OG1 was added to the drinking water for another 10 days. Mice in the control group received 5×10^7 cfu/mL of the mutant positive for AS and positive for BS but were not treated with antibiotics, with the exception of an initial 10-day course of vancomycin at 1.0 g/L for decolonization of indigenous enterococci. The bacteria were derived from fresh overnight cultures containing 10 mg/L chloramphenicol and an additional 10 mg/L tetracycline for the BS-negative mutants. The bacteria were washed three times with sterile water before they were added to the drinking water together with the antibiotics, and the contaminated drinking water was replaced every other day. After this colonization period, the animals received sterile water overnight and then were killed by CO₂ suffocation. Their abdomens were opened aseptically, and 0.1 mL of blood was obtained by heart puncture. Parts of the left liver lobe, the spleen, and mesenteric lymph nodes, in that order, were removed and weighed, homogenized, and diluted in tryptic soy broth containing 0.5 g/L polyanetholesulfonic acid (Sigma Chemical, St. Louis, MO). Appropriate dilutions of all samples were spread on tryptic soy agar plates with 5% sheep blood (Becton Dickinson), on selective plates for enterococci, and on MacConkey agar. The minimum detection threshold was 10² cfu/g. Bacterial counts were determined from the selective plates after 48 hrs of incubation at 37°C, and the appropriate phenotypes were confirmed by further subculture of single colonies using selective antibiotics and by the clumping reaction in appropriate combinations. In cases of high numbers of enterococci—which especially was the case in cultures derived from mesenteric lymph nodes—ten single colonies were selected randomly for confirmatory testing. After the organs were removed, the cecum was opened and 100 mg of cecal contents was diluted in phosphate-buffered saline. Parts of the specimens were immediately taken to an anaerobic chamber for further processing and anaerobic culture on blood agar with kanamycin and vancomycin and on Brucella agar with 5% sheep blood

(both PML Microbiologicals, Mississauga, ON, Canada). The remaining specimens were cultured aerobically on Enterococcosel and MacConkey agar. To verify that the treatment with vancomycin effectively removed indigenous enterococci from the mouse intestinal flora, an additional set of three mice were investigated. They were handled with barrier precautions as described previously, and their pellets were examined every 2 days for the presence of enterococci, while they were treated with vancomycin for 10 days, which was followed by a 10-day course of sterile water. Then they were killed, and segments of their colon, cecum, and jejunum were swabbed and cultured for the presence of enterococci on selective plates after enrichment in tryptic soy broth.

Statistical Analysis. The presence of enterococci in the organs was compared by Wilcoxon's test, and analysis of variance was calculated for comparison of log₁₀ of viable counts of enterococci per gram recovered from mesenteric lymph nodes with the use of Bonferroni posttests to compare the different groups (GraphPad Prism3, GraphPad Software, San Diego, CA). The association of enterococci recovered from lymph nodes with retrieval from livers or spleens was calculated by logistic regression analysis using R (www.r-project.org).

RESULTS

Intestinal Colonization and Validity of the Animal Model. The initial fecal samples yielded *Enterobacteriaceae* (up to 10⁸ cfu/g feces) and up to 10⁶ cfu/g indigenous enterococci. After a maximum of 4 days of antibiotic treatment, no more enterococci were found in any of the mice until contamination of the drinking water with the enterococcal mutant strains was begun. Likewise, the specimens of the colon, cecum, and jejunum of the mice treated with vancomycin, which was followed by a 10-day course of sterile water, were free from any enterococci, thus confirming effective decolonization of indigenous enterococci in our animal model. During induction of intestinal overgrowth, the enterococcal strains became established within 2 days, and the concentration in fecal pellets ranged from 10⁷ to 10⁹ cfu/g in all groups. After kill, the concentration of the enterococcal mutants cultured from cecal contents ranged from 2 to 6×10^9 cfu/g, and no statistically significant differences between the groups were found. In all mice treated with antibiotics during the intestinal overgrowth phase, the cecal contents were devoid of *Enterobacteriaceae*, and only the specific enterococcal strains grew on the plates cultured under anaerobic conditions. In the mice without fur-

ther antibiotic treatment, *Enterobacteriaceae* and anaerobes were found at 10^8 and 10^9 cfu/g feces, respectively.

Extraintestinal Translocation of *Enterococci*. Figure 1 shows the mean cfu per gram of mesenteric lymph nodes for the different enterococcal mutants (range for positive lymph nodes: 1.6×10^2 to 9.7×10^3 /g). No other bacterial species were detected in any organ sample. Wilcoxon's test was used to compare the groups of mice with and without antibiotic treatment inoculated with the identical mutant of *E. faecalis* OG1SSp harboring the plasmid pINY1801 (AS+ and BS+). The cfu in mesenteric lymph nodes was significantly lower in the mice that were not treated with antibiotics ($p = .016$). Multigroup analysis of variance demonstrated a statistically significant difference between this group and each of the antibiotic-treated groups inoculated with the different bacterial mutants ($p < .05$ and $p < .01$, respectively; Table 1). However, in the antibiotic-treated animals, no significant difference was found between any of the different mutants.

No enterococci were recovered from the blood of any of the mice. The overall frequency of recovery of enterococci from livers and spleens was 14% and 10%, respectively, and no differences were found between the groups. A positive culture from either spleen or liver was more likely in mice from which enterococci

were recovered from the mesenteric lymph nodes (28% vs. 12%, rate ratio = 2.39). However, since organ involvement was a rare event overall, this result was not statistically significant ($p = .30$ by logistic regression analysis).

DISCUSSION

In our mouse model, we found that oral antibiotics can select for extraintestinal translocation of *E. faecalis*. Neither of the previously described enterococcal virulence factors, AS and BS, seems to be required for this process.

Animal experiments, as well as studies using intestinal cell lines, such as Caco-2 or HT-29, have demonstrated that enterococci are able to translocate through the intact gut mucosal layer (9, 11, 14, 15). Although the precise molecular mechanisms remain obscure, it has conclusively been shown that the normal intestinal flora and especially anaerobic bacteria—which normally constitute the majority of gut microorganisms—play a pivotal role in limiting the translocation of potential pathogens *in vivo* (14, 16). This is especially important since translocation of enteric bacteria into the mesenteric lymph nodes is considered the first step in extraintestinal dissemination, which can result in serious and life-threatening diseases, such as abscess formation, bacteremia, and sepsis (17).

Wells et al. (14) tested the effect of clindamycin and metronidazole, two antibiotics with good activity against anaerobes, in a mouse model of intestinal colonization and translocation. During treatment of the animals with metronidazole, enterococci were the predominant Gram-positive species in the cecal flora. In 26% of the experimental animals, enterococcal colonization was followed by translocation into mesenteric lymph nodes (14). These results are corroborated by the clinical observation of Edmond et al. (5), who found that the use of antimicrobial agents with significant activity against anaerobes (such as metronidazole, clindamycin, and imipenem) was a risk factor for the development of bacteremia due to vancomycin-resistant enterococci, especially in neutropenic patients. Furthermore, several clinical studies linked the use of cephalosporins to enterococcal bacteremia (18, 19).

The animal model described in the present study relies on these findings by using metronidazole and cefoxitin, which exert strong activity against anaerobes and to which enterococci are intrinsically resistant. With this treatment regimen, we were able to effectively eliminate the indigenous intestinal aerobic and anaerobic microflora and establish *E. faecalis* as the only colonizing bacteria. Even though we cannot draw any conclusions from our experiments as to whether the elimination of anaerobes or of coliform bacteria was more important, the translocation to mesenteric lymph nodes was significantly increased compared with that in animals without antibiotic treatment. We also cannot comment on the exact pathway of translocation, but at least a gross loss in intestinal barrier function could be excluded by light microscopy (data not shown). However, in such juvenile animals as used in our study and in other investigations (11, 14, 16), enterocytosis of bacteria via enteral M-cells is important in accomplishing direct contact of the immune system with gut-derived antigens (20). This means that our findings might not necessarily be identical in adult animals, when the indigenous gut microflora has been established more firmly and the ability of pathogens to undergo M-cell transport might well be different. Furthermore, in the otherwise immunocompetent mice used in this study, further dissemination to the liver and spleen was rarely seen and enterococci were never recovered from the bloodstream. Therefore, we can

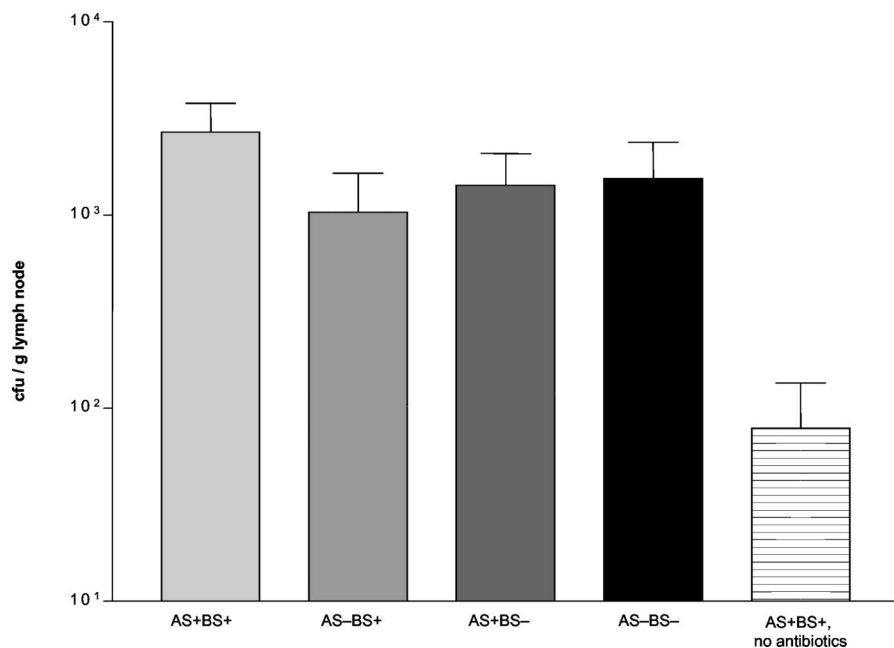


Figure 1. Comparison of colony-forming units (cfu)/g of lymph nodes (mean, SEM) for the five groups of mice with intestinal overgrowth by isogenic mutants of *E. faecalis* expressing (+) or not expressing (–) aggregation substance (AS) or binding substance (BS).

Table 1. Characteristics of the different groups of mice and translocation frequency of *Enterococcus faecalis*

Phenotype of Enterococcal Mutant ^a	Antibiotic Treatment	Incidence of <i>E. faecalis</i> Translocation to Mesenteric Lymph Nodes, %	Incidence of <i>E. faecalis</i> Translocation to Livers, %	Incidence of <i>E. faecalis</i> Translocation to Spleens, %
AS+, BS+	Yes	67 ^b	22	22
AS-, BS+	Yes	60 ^b	0	0
AS+, BS-	Yes	90 ^c	20	10
AS-, BS-	Yes	90 ^c	0	20
AS+, BS+ (control group)	No	20	20	0

^aAS+/AS-, mutant expresses/does not express enterococcal aggregation substance; BS+/BS-, mutant expresses/does not express enterococcal binding substance; ^b*p* < .05; ^c*p* < .01 compared with control group.

only speculate about the importance of the microflora and the putative virulence factors AS and BS for subsequent dissemination of enterococci from mesenteric lymph nodes to the bloodstream, which has been described in neutropenic animals after treatment with cyclophosphamide (21). Nevertheless, these experimental data contribute to our understanding of the epidemiologic link between certain antibiotics and severe enterococcal infections and do not simply reflect an association with drugs used in critically ill patients (5). Rather, these data point to a pathogenic principle that may be implemented in our decisions for antibiotic treatment in a clinical setting (22).

Our animal model modifies the original method by replacing the application of antibiotics via intramuscular injection and oral inoculation of enterococci via a feeding needle (11). Instead, we established a decolonization period by adding vancomycin, gentamicin, cefoxitin, and metronidazole to the drinking water and were able to prevent recolonization by regular replacement of the animal cages and by use of strict barrier precautions. Likewise, we added the specific enterococcal strains to the drinking water and administered cefoxitin and metronidazole continuously during the subsequent colonization period. Furthermore, we clearly excluded confounding effects by indigenous enterococci, since there were no enterococci in fecal pellets or in the gut segments of the mice that were treated with vancomycin, which was followed by a 10-day course of sterile water. Therefore, the method presented here is less invasive and less labor intensive than the original method and enabled us to assess multiple comparisons of virulence factors that might be involved in translocation and dissemination of *E. faecalis*.

The importance of AS as a virulence factor in enterococcal infections has been

investigated repeatedly in animal models and *in vitro*. Although good evidence supports the role of AS together with BS as a virulence factor in endocarditis (13, 23, 24), the roles of these factors in animal models of peritonitis and endophthalmitis are less clear (25, 26). There is evidence that AS, besides functioning as a component of the pheromone-responsive genetic exchange system (12, 27, 28), mediates binding to eukaryotic cell surface structures such as renal tubular cells and cultured intestinal epithelial cells (9, 29–31). This interaction was further confirmed after heterologous expression of AS in *Lactococcus lactis* (32). Furthermore, AS appears to promote intracellular survival of enterococci inside human neutrophils (33), entry into human macrophages (34), and intracellular uptake into colonic mucosal cells in an *ex vivo* model (8). Such interactions mostly have been attributed to the presence of RGD motifs (arginine, glycine, and aspartic acid) within the amino acid sequence of AS. Whereas RGD motifs are rather uncommon in bacteria (35), they have important functions in eukaryotic organisms for binding of extracellular proteins, such as fibronectin, to integrins of cell membranes (36). However, neither of the studies investigated the confounding effects of BS, since processing of autoaggregated clumps of enterococci by epithelial cells might be different from that of unaggregated cells. Furthermore, no prior animal experiments have been performed to elucidate the role of AS in the process of enterococcal translocation *in vivo*.

Our results focused on the effects of AS and BS on translocation of *E. faecalis* from the gastrointestinal tract *in vivo*. Analyzing a set of four different mutants, we were able to investigate the effect of AS and BS on translocation independently and thereby exclude the effect of

autoaggregation of enterococci as a confounding factor. In contrast to the previously mentioned evidence from studies *in vitro*, our results demonstrate that neither AS or BS alone nor both factors together promote the translocation of *E. faecalis* compared with an isogenic AS/BS-negative strain. This indicates that the interaction between AS and intestinal epithelial cells *in vitro* might not be clinically relevant to effective translocation of bacteria into the mesenteric lymph nodes. One limitation might be that we investigated only one control group with respect to antibiotic treatment. However, this group was fed with *E. faecalis* expressing the putative virulence factors AS and BS together, but the translocation of *E. faecalis* to mesenteric lymph nodes was significantly less compared with all other groups with antibiotic treatment. The higher incidence of AS in *E. faecalis* isolated from bloodstream and other infections (7, 37) may be due to other properties of AS, but our results do not support the hypothesis that AS facilitates the translocation of *E. faecalis in vivo*.

CONCLUSION

Oral treatment with the antianaerobic antibiotics metronidazole and cefoxitin promoted the translocation of *E. faecalis* into mesenteric lymph nodes of immunocompetent mice. This process was not enhanced by the presence of the enterococcal virulence factors AS or BS. The high reproducibility of the experiments is encouraging for further exploration of host and microbial factors contributing to translocation and may provide a better understanding of the pathogenesis of enterococcal infections in patients in intensive care units.

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