Role of Anaerobic Flora in the Translocation of Aerobic and Facultatively Anaerobic Intestinal Bacteria

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It is thought that the normal enteric microflora acts not only to prevent intestinal colonization but also to prevent subsequent systemic dissemination of ingested, potentially pathogenic bacteria. To determine the relative roles of specific components of the intestinal bacterial flora in bacterial translocation out of the gut, mice were given various antimicrobial agents to selectively eliminate specific groups of intestinal bacteria. The cecal flora and the translocating bacteria in mesenteric lymph nodes were monitored both before and after oral inoculation with antibiotic-resistant Escherichia coli C25. Orally administered streptomycin selectively eliminated cecal facultative gram-negative bacilli, orally administered bacitracin-streptomycin eliminated all cecal bacterial species except low numbers of aerobic sporeformers, and parenterally administered metronidazole selectively eliminated cecal anaerobic bacteria. Compared with control mice, only metronidazole-treated mice had significantly increased rates of dissemination of intestinal bacteria into mesenteric lymph nodes, indicating that the exclusive absence of anaerobic bacteria facilitated the translocation of the intestinal facultative bacteria. In a parallel experiment with streptomycin-resistant E. coli C25 as a marker, parallel results were obtained. Metronidazole increased the translocation of the marker strain and the indigenous strains of intestinal bacteria. Thus, anaerobes appeared to play a key role in confining indigenous bacteria to the gut. However, intestinal colonization and translocation of E. coli C25 occurred most readily after bacitracin-streptomycin treatment, suggesting that in addition to anaerobic bacteria, other bacterial groups may play a role in limiting the intestinal colonization and extraintestinal dissemination of E. coli C25.

The normal intestinal microflora contains a relatively stable population of more than 500 species of bacteria (14). This population of indigenous bacteria not only is stable, but also functions to exclude intestinal colonization by nonindigenous microbes (1, 5, 11, 13, 18). Many studies have shown that intestinal colonization by exogenous pathogens can be facilitated by antibiotic therapy designed to upset the integrity of the normal bacterial flora (2, 10, 11, 18, 21–23). Successful intestinal colonization has been shown to facilitate extraintestinal dissemination of potentially pathogenic bacteria (1, 3, 11, 19, 20, 23).

Of all the enteric bacterial groups, anaerobic bacteria seem to play a key role in preventing the enteric colonization of exogenous bacteria by the process of “colonization resistance,” a term introduced by Van der Waaij et al. (21–23). The hypothesis of colonization resistance has been applied to clinical medicine. By this hypothesis, patients susceptible to opportunistic enteric pathogens will be best protected by the selective elimination of intestinal facultative gram-negative bacilli, while the indigenous anaerobic microflora are preserved. In clinical practice, this type of selective antimicrobial modulation of the intestinal microflora has been associated with a decreased incidence of infectious episodes in immunosuppressed patients (4, 7–9, 24). There has been no direct experimental proof, however (in either humans or animals), that the anaerobic flora plays an important role in confining potential pathogens to the gut. The present studies were designed to compare the translocation of viable bacteria from the enteric lumen to the mesenteric lymph nodes (MLN) in the presence or relative absence of intestinal anaerobic bacteria.

MATERIALS AND METHODS

Selective intestinal decontamination and oral inoculation of mice with Escherichia coli C25. Separate groups of female 18- to 22-g Swiss Webster mice (Bio-Lab Corp., St. Paul, Minn.) were given one of three types of antimicrobial therapy designed to selectively eliminate different populations of intestinal bacteria. In an attempt to eliminate facultative gram-negative bacilli, mice were given 2 mg of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml in the drinking water. In an attempt to eliminate all species of intestinal bacteria, another group of mice was given 2 mg of bacitracin (Sigma) plus 2 mg of streptomycin per ml in the drinking water. In an attempt to selectively eliminate all species of strictly anaerobic bacteria, metronidazole (Searle Pharmaceuticals, Inc., Chicago, Ill.) was administered. Preliminary experiments utilized 4 mg of metronidazole per ml in the drinking water and confirmed previous reports of others who have noted that orally administered metronidazole does not eliminate intestinal anaerobic bacteria in rodents (2, 10). Mice were therefore given intramuscular metronidazole twice a day in a dose of 4 mg/0.2 ml per mouse. Control mice were given normal drinking water and intramuscular twice-daily injections of phosphate-buffered saline. Groups of mice were sacrificed after day 3 of drug therapy, and cecal and MLN bacteria were cultured as described below.

After day 3 of drug therapy, cohort groups of mice were orally inoculated with E. coli C25 (provided by Rodney Berg, Louisiana State University, Shreveport). This streptomycin-resistant strain was originally a clinical isolate and has been used in several rodent studies monitoring the translocation of intestinal bacteria (3, 12, 25). Stock cultures...
were maintained at -20°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% glycerol. A feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.) was used to orally inoculate mice with 0.1 ml of approximately 10^10 saline-washed E. coli C25 from an over-night brain heart infusion broth culture. Mice were sacrificed 48 h later, and cecal and MLN bacteria were again cultured as described below. Antibiotic treatment was continued for the duration of the experiment.

Enumeration of viable MLN and cecal bacteria. Each mouse was killed by cervical dislocation, and the MLN was excised prior to excision of the cecum. Tissues were processed aseptically.

A ground-glass stopper was used to homogenize each MLN in 2 ml of phosphate-buffered saline with 0.1% gelatin (6), and 100 μl was plated in duplicate onto colistin-nalidixic agar (Difco) for enumeration of aerobic and facultative gram-positive bacteria, MacConkey agar (Difco) supplemented with 10% lactose (Sigma) for enumeration of aerobic and facultative gram-negative bacilli, and MacConkey agar without lactose but supplemented with 100 μg of streptomycin per ml for enumeration of streptomycin-resistant, facultative gram-negative bacilli. Cage was taken to identify and eliminate from the facultative gram-negative cultures such gram-positive species as enterococci which can grow on MacConkey agar but were always included in the enumeration of gram-positive bacteria. Agar media were incubated aerobically at 35°C for 24 to 48 h. MLN bacteria were enumerated as the total number of viable bacteria per MLN, and statistical significance was assessed by the chi-square test. The MLN data presented herein represent pooled data from similar replicate experiments with 9 or 10 mice per experimental group.

Ceca were aseptically excised, weighed, and immediately transferred to an anaerobic chamber (Forma Scientific, Marietta, Ohio), and Waring blenders were used to homogenize tissues in prereduced Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.05% cysteine hydrochloride (Sigma). The homogenate was serially diluted in the same solution and plated onto Wilkins-Chalgren agar (Difco), supplemented with 100 μg of gentamicin per ml (LyphoMed, Inc., Melrose Park, Ill.), which allowed growth of strictly anaerobic bacteria but inhibited growth of facultative bacteria. Cecal homogenates were also plated onto the media described above for processing MLN homogenates. Preliminary experiments showed that the same numbers of anaerobic bacteria were recovered on Wilkins-Chalgren agar supplemented with gentamicin as were recovered on unsupplemented Wilkins-Chalgren agar. The supplemented Wilkins-Chalgren agar was incubated anaerobically at 35°C for 48 to 72 h and was used to enumerate the total numbers of strictly anaerobic bacteria. The other agar media were incubated as described above for MLN homogenates. Cecal bacteria were enumerated as the viable log_{10} per gram (wet weight) of cecum, and the limit of detection of the assay was 500 bacteria per cecum. The data presented herein represent pooled data from similar replicate experiments with four mice in each experimental group.

Identification of bacteria. Bacteria were identified by standard techniques with facultative gram-negative bacilli identified primarily by the API 20E system (Analytab Products, Plainview, N.Y.).

Histological sections of intestinal tissue. Intestinal tissues were excised after day 3 of drug therapy. Duodenal tissue (5 cm proximal to the stomach), ileal tissue (5 cm proximal to the cecum), and cecal tissue (4 cm distal to the cecal tonsil) were placed in Hollande-Bouin fixative for 48 h and then transferred to buffered Formalin until processed for histological sections. Tissue sections were stained with hematoxylin-eosin stain and were processed from three animals in each drug treatment group.

RESULTS

Effect of orally administered streptomycin or bacitracin-streptomycin or parenterally administered metronidazole on mouse cecal flora and microscopic anatomy. Mice were sacrificed after day 3 of drug therapy. No histological intestinal changes were noted. Also, no gross pathologic changes were noted in any of the treatment groups, except that the cecal size was macroscopically increased in response to antibiotic therapy (Table 1). This increase was most evident in mice treated with bacitracin-streptomycin, which was also the group with the greatest alteration in cecal flora, as explained below.

After day 3 of drug therapy, mice were sacrificed and the cecal bacterial flora was characterized. Two of eight control mice had low levels of streptomycin-resistant, facultative
gram-negative bacilli. Streptomycin-treated mice had no detectable facultative gram-negative bacilli other than the relatively low levels of streptomycin-resistant, facultative gram-negative bacilli recovered from two of eight mice (Table 1). Bacitracin-streptomycin treatment effectively eliminated all cecal microbes except low numbers of aerobic sporeformers, i.e., Bacillus spp. and fungi. Metronidazole treatment selectively eliminated all strictly anaerobic bacteria; mice so treated also had a 100-fold increase in aerobic and facultative bacteria and exhibited the emergence of indigenous streptomycin-resistant facultative bacilli (Table 1).

**Effect of oral inoculation of antibiotic-resistant E. coli C25 on cecal flora of mice treated with streptomycin, bacitracin-streptomycin, or metronidazole.** After day 3 of drug therapy, each mouse was orally inoculated with $10^{10}$ streptomycin-resistant E. coli C25. Approximately $10^2$ E. coli C25 (approximately 99.9999%) were eliminated from control mice within 48 h (Table 1). In all groups of drug-treated mice, E. coli C25 was able to persist in the intestinal tract at levels of approximately $10^3$ to $10^5$E. coli C25 was recovered within 48 h (Table 1). After oral inoculation with E. coli C25, the cecal streptomycin-resistant gram-negative bacilli consistently appeared to be a pure culture of E. coli C25.

**Effect of orally administered streptomycin or bacitracin-streptomycin or parenterally administered metronidazole on translocation of indigenous intestinal bacteria.** Control mice, streptomycin-treated mice, and bacitracin-streptomycin-treated mice had low levels of bacterial translocation into MLN; i.e., only an occasional mouse had indigenous bacteria recovered from MLN (Table 2). However, in sharp contrast, indigenous, facultative intestinal bacteria were isolated from the majority of mouse MLN in the metronidazole treatment group; here, the translocating bacteria were primarily enterococci and indigenous E. coli that were streptomycin sensitive (Table 2).

**Effect of orally administered streptomycin or bacitracin-streptomycin or parenterally administered metronidazole on translocation of antibiotic-resistant E. coli C25.** To study the effect of intestinal overgrowth of E. coli on bacterial translocation into MLN, drug-treated mice were orally inoculated with $10^6$ streptomycin-resistant E. coli C25. As mentioned above, control mice had eliminated much of the E. coli C25 inoculum by the time of sacrifice (Table 1), and this organism was not recovered from any MLN. That time, only 1 of 19 control mice had bacteria cultured from MLN, and the isolate was identified as coagulase-negative Staphylococcus sp. (Table 3). E. coli C25 was cultured from the MLN of 50% (9 of 18) of streptomycin-treated mice and from nearly all MLN from bacitracin-streptomycin-treated mice (Table 3). Metronidazole-treated mice were the only group that had translocation of E. coli C25 with simultaneous translocation of indigenous intestinal flora (Table 3).

**TABLE 2. Effect of selective intestinal decontamination on the translocation of indigenous intestinal bacteria into MLN**

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Intestinal bacteria decreased or eliminated</th>
<th>No. of mice with bacteria in MLN/total no. of mice</th>
<th>No. and identity of translocating species in individual mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>1/20</td>
<td>20 E. coli</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Facultative gram-negative bacilli</td>
<td>2/20</td>
<td>20 E. coli&lt;sup&gt;a&lt;/sup&gt;; 1.310 group D streptococci (not enterococci)</td>
</tr>
<tr>
<td>Bacitracin-streptomycin</td>
<td>All species except sporeformers</td>
<td>0/20</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>Strict anaerobes</td>
<td>13/19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;2 × 10&lt;sup&gt;4&lt;/sup&gt; mixed flora: 20 E. coli; 10 E. coli + 50 enterococci; 10 E. coli + 20 enterococci; 10 E. coli + 40 enterococci; 10 enterococci; 10 E. coli: 10 Proteus sp.: 10 enterococci; 20 E. coli + 20 enterococci; 50 E. coli + 60 enterococci; 50 E. coli + 60 enterococci; 20 E. coli + 60 enterococci</td>
</tr>
</tbody>
</table>

<sup>a</sup>See Table 1, footnote a.

<sup>b</sup>All E. coli were streptomycin sensitive except this isolate.

<sup>c</sup>One mouse died before sacrifice. Significant increase compared with control group; P < 0.01.

**DISCUSSION**

The concept of colonization resistance had its origins in the early 1970s. At that time, Van der Waaij et al. (21–23) noted that the administration of oral antibiotics resulted in a decrease in the number of bacteria required to successfully colonize mice that had been orally inoculated with various species of antibiotic-resistant bacteria. In comparing streptomycin- and ampicillin-treated mice, it was also noted that fewer bacteria were needed to colonize mice treated with parenterally administered streptomycin than those treated with parenterally administered ampicillin (21). Consequently, Van der Waaij et al. (21) speculated that because ampicillin (but presumably not streptomycin) was excreted in the bile, intestinal drug concentrations might not be the key mechanism by which oral drug therapy modulated the ability of an organism to colonize the intestinal tract; rather, the primary mechanism by which a drug facilitated intestinal colonization was possibly due to its ability to eliminate mucosa-associated anaerobic bacteria. Contrary to this theory, recent studies with mice (10) have shown that the oral administration of streptomycin results in high intestinal drug levels, while ampicillin cannot be detected, presumably due to its inactivation by beta-lactamase-producing gut bacteria (19). However, additional studies by Van der Waaij et al. with gnotobiotic mice colonized only with anaerobic bacteria...
indirectly confirmed that anaerobic bacteria functioned to prevent intestinal colonization by orally administered bacterial pathogens (22). Van der Waaij et al. (21-23) therefore defined colonization resistance as a mechanism which apparently controls the microbial colonization of mice and in which anaerobic species play a major role. As mentioned previously, the clinical applicability of this theory has been widely documented in immunsuppressed humans (4, 7-9, 24).

Although the reports of Van der Waaij et al. (21-23) emphasized the pivotal role of the anaerobe in colonization resistance, Hentges et al. (10) indirectly indicated that other groups of intestinal bacteria might also function to prevent intestinal colonization by potential pathogens. Because the role of the anaerobe in colonization resistance is a clinically important issue that often dictates the design of prophylactic therapy in immunocompromised patients, it is important to clarify the role of the anaerobic bacteria in facilitating or preventing systemic infection by normal gut bacteria. To date, no study has been reported that involves selective elimination of all species of intestinal anaerobes to determine their role in colonization resistance. We have therefore attempted to design such a study to directly analyze the role of the anaerobe in (i) confining indigenous gut bacteria, (ii) preventing intestinal colonization by an exogenous potential pathogen, i.e., *E. coli*, and (iii) preventing extraintestinal dissemination of this orally inoculated strain of *E. coli*.

Although intestinal bacterial population levels have not been strictly defined, control mice apparently harbored normal population levels of intestinal aerobes, anaerobes, and enteric bacilli (2, 15). After antibiotic therapy, only two of eight ceca from control mice contained a few streptomycin-resistant, facultative gram-negative bacilli (Table 1). This study indicated that the animal colony harbored some streptomycin-resistant enteric bacilli that could possibly replicate to high population levels during streptomycin therapy. In addition, streptomycin selectively eliminated cecal facultative gram-negative bacilli in all but two of the eight mice studied. This selective elimination did not appear to be a critical problem for the experimental design, because in these two mice, streptomycin therapy still reduced the numbers of cecal enteric bacilli by at least 100-fold; i.e., streptomycin-resistant enteric bacilli did not appear to overgrow in the intestinal tract. Streptomycin therapy likewise did not cause an overall increase in the rate of translocation of intestinal bacteria into MLN compared with the typically low levels observed in control animals (Table 2). This lack of increase was to be expected because streptomycin eliminated facultative gram-negative bacilli, and these organisms are the most frequently reported species of translocating bacteria (2, 3, 12, 17). One streptomycin-treated mouse had translocating *E. coli* recovered from MLN, and this indigenous isolate was streptomycin resistant (Table 2). The intestinal flora of this particular mouse was not studied, but perhaps this animal contained high intestinal population levels of an indigenous strain of streptomycin-resistant *E. coli* that overgrew in response to streptomycin therapy. However, in general, the data from streptomycin-treated animals indicated that this drug eliminated or reduced the numbers of cecal enteric bacilli and did not increase bacterial translocation into MLN.

Bacitracin-streptomycin therapy eliminated all viable cecal bacteria except occasional sporeformers such as *Bacillus* spp. and fungi (Table 1). Treated mice did not have any translocating intestinal bacteria recovered from MLN (Table 2). This was an expected result because bacitracin-streptomycin treatment eliminated all the usual (2, 3, 12, 17) translocating species from the intestinal tract and there was no evidence of the emergence of resistant strains in this animal model.

Berg (2) has reported that adding metronidazole to the drinking water of mice produced no decrease (and in fact produced a slight increase) in the numbers of cecal anaerobic bacteria. In the Berg study (2), the gram-negative enteric flora increased about 1,000-fold and was accompanied by an increased rate of translocation of enteric bacteria into MLN.
In a recent study, however, Hentges et al. (10) added metronidazole to the drinking water of mice and reported no detectable cecal concentration of drug and no increase in the translocation of intestinal bacteria into MLN. In this latter study, the intestinal flora was not characterized, so it is difficult to compare results with those of Berg. Our preliminary experiments (data not presented) confirm that orally administered metronidazole had essentially no effect on the total numbers of cecal anaerobic bacteria. During an attempt to eliminate intestinal anaerobes, we subsequently found that intramuscular metronidazole selectively eliminated all detectable, strictly anaerobic cecal bacteria. The present experiments confirmed that finding. In this animal model, parenterally administered metronidazole eliminated all strict anaerobes while permitting the intestinal overgrowth of aerobic and facultative species (Table 1). Onderdonk et al. (16) reported that parenterally administered metronidazole appeared to have an in vivo effect on the killing of facultative bacteria in a rat model of intra-abdominal infection. In our animal model, parenterally administered metronidazole did not appear to inhibit the growth of facultative bacteria, as indicated by a 10-fold increase in the numbers of cecal facultative gram-positive and gram-negative bacteria in metronidazole-treated mice (Table 1). This overgrowth of facultative bacteria was accompanied by a significant increase in the rate of translocation of these indigenous intestinal bacteria (Table 2). These results support the hypothesis that anaerobic bacteria play a critical role in preventing the extraintestinal dissemination of normal, indigenous gut bacteria.

Parallel results were obtained in mice colonized with E. coli C25. Mice with >10^8 E. coli C25 per g of cecum demonstrated translocation of this organism. If lower numbers of E. coli C25 were found in the cecum (as noted in control mice), no translocation could be demonstrated. After metronidazole treatment, E. coli C25 consistently persisted in the cecum at concentrations of 10^8 to 10^9/g (Table 1), which was a somewhat lower concentration than the extremely high population levels of 10^9.5 to 10^11 recovered from bacitracin-streptomycin-treated mice. Because metronidazole-treated mice had only anaerobic bacteria eliminated from the cecal flora, their relatively low cecal concentration of E. coli C25 indicated that aerobic or facultative bacteria had some role in limiting the intestinal colonization of the orally inoculated E. coli C25. This observation suggested that, in addition to anaerobic bacteria, other bacterial species had a role in colonization resistance. The incidence of translocation of E. coli C25 in metronidazole-treated mice was comparable with that of the streptomycin group but was consistently lower than that of the bacitracin-streptomycin group (Table 3). This also indicated that, in addition to anaerobic bacteria, other groups of bacteria functioned to prevent the extraintestinal dissemination of E. coli C25. It should also be noted that in metronidazole-treated mice, intestinal colonization and translocation of E. coli C25 did not appear to affect the rate of translocation of indigenous flora. In fact, it could be argued that E. coli C25 appeared to be carried out of the intestinal tract along with the indigenous flora, or vice versa (Table 3).

The results of this study partially confirmed the proposed theory of colonization resistance because anaerobic bacteria played a major role in preventing the translocation of indigenous gut bacteria and in preventing intestinal colonization by an exogenous pathogen, namely, E. coli C25. However, the results of this study also suggest that in addition to the anaerobe, other groups of bacteria had a role in preventing intestinal colonization by E. coli C25 and in preventing the extraintestinal dissemination of E. coli C25.

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