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Colonization Resistance of the Human Intestinal Microflora: Testing the Hypothesis in Normal Volunteers

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Colonization resistance is the mechanism whereby the intestinal microflora protects itself against incursion by new and often harmful microorganisms. Some authors have claimed that colonization resistance is related to the integrity of the anaerobic flora, but this point has not been established in humans. In previous studies in our laboratory cefoxitin, piperacillin, cefoperazone or aztreonam were administered intravenously to healthy volunteers in order to study changes in the intestinal flora and acquisition of new strains. Seven of 16 antibiotic-treated subjects were colonized with gram-negative bacilli, but no correlation was observed between this colonization and the suppression of either anaerobes or any other component of the fecal flora. Marked strains of *Escherichia coli* and *Pseudomonas aeruginosa* were also administered by mouth in order to test acquisition of new bacteria. The fed bacteria were found in the stools of both antibiotic-treated and control subjects; the antibiotics had no apparent influence on the ability of these strains to colonize the intestinal tract. Our work, along with findings of others, supports the concept that colonization resistance occurs in humans and is diminished by antibiotic administration. However, it does not support the hypothesis that colonization resistance is related to the anaerobic microflora.

With the advent of broad spectrum antimicrobial drugs it has become apparent that their suppressive activities are exerted not only against invading pathogens but against the host's resident microflora as well (1–6). As a result, the flora undergoes profound changes, particularly apparent with the overgrowth of antibiotic-resistant bacteria and yeasts (5–10).

The normal intestinal microflora possesses a protective mechanism against the incursion of unusual and potentially harmful organisms. Known as "colonization resistance" (11), this protective factor has been related by some authors to the anaerobic component of the normal flora (11–14), while other authors have ascribed this mechanism to facultative gram-negative rods (15–18). Most studies, however, have been carried out in animals, and there are few data in humans to investigate which components of the microflora are responsible for colonization resistance.

In this paper we review previous work carried out by our group and by other investigators. Our studies, which have been published previously in greater detail (19, 20), examined the effects of several beta-lactam antibiotics on the normal intestinal microflora in healthy volunteers. The major focus was to discover whether the anaerobes, as claimed by some workers, or other groups of organisms were involved in this protective mechanism.

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Materials and Methods

Twenty healthy volunteers who had received no antibiotic treatment within the preceding month were randomized to receive one of the following regimens intravenously: 2 g aztreonam every 6 h, 2 g cefoperazone every 12 h, 2 g cefoxitin every 6 h, or 3 g piperacillin every 4 h. Each regimen was given to four subjects for nine days in the Clinical Study Unit which is located in one of the wards of New England Medical Center. Four control subjects did not receive antibiotics but were required to eat at least two meals in the Clinical Study Unit each day for eight days.

Stool Specimens for Microbiological Studies. Stool specimens were collected within one week before antibiotic treatment, on days 3, 6 and 9 of treatment, after antibiotics were discontinued (on days 12 and 14), and then twice weekly until the stool cultures returned to their pre-treatment composition.

All specimens were taken immediately to the laboratory and processed within 1 h. One gram of the specimen was transported to an anaerobic chamber, diluted 10-fold in phosphate buffered saline plus 1% gelatin (PBS + Gel), and emulsified in a Vortex mixer. Serial 10-fold dilutions were made in PBS + Gel. Aliquots of 0.1 ml of selected dilutions were plated on the media indicated in Table 1.

Table 1: Media used for microbiological studies.

Medium	Purpose
Brucella blood agar + vit K, hemin	total aerobic and anaerobic counts
MacConkey agar	*Enterobacteriaceae*
Kanamycin esculin azide agar	enterococci
Salt mannitol agar	staphylococci
Pseudosel	pseudomonads
Mycosel	yeasts
Kanamycin-vancomycin laked blood agar	*Bacteroides fragilis* group
Cycloserine-cefoxitin-fructose agar	*Clostridium difficile*

Assay of Cytotoxin. All stool specimens were assayed for *Clostridium difficile* cytotoxin by means of a tissue culture assay with neutralization by specific antitoxin (21).

Administration of Colonizing Organisms. On day 8 of treatment 10^{10} CFU of *Escherichia coli* and 10^7 CFU of *Pseudomonas aeruginosa* were administrered together in 30 ml of chocolate milk. This was repeated on day 12 (3 days after the end of treatment). The control subjects were fed these strains only once, eight days after they started eating their meals in the Clinical Study Unit.

For ethical reasons bacterial strains were chosen which were widely sensitive to antibiotics including those administered in this study. The *Escherichia coli* was a K12 C600 nalidixic acid-resistant mutant strain. The MICs of the drugs (μg/ml) in the study were as follows: aztreonam 0.5, piperacillin 4, and cefoxitin 8. The ability of this strain to grow on a medium containing 100 μg/ml of nalidixic acid was used for its detection in stool. The *Pseudomonas aeruginosa* strain was a clinical isolate; the MICs were aztreonam 4, piperacillin 8, cefoperazone 16, and cefoxitin 256. It was serogroup-14, according to the *Pseudomonas aeruginosa* antiserum set (Difco Laboratories, USA), and this unusual epidemiological character was used for its identification.

Microflora Counts. Plates were incubated at 37 °C for 24 h in the case of aerobic cultures or for 48 h in an anaerobic chamber in the case of anaerobic cultures. Plates that showed 30 to 300 colonies were used for bacterial counts. The fed strain of *Escherichia coli* was counted on plates containing 100 μg/ml nalidixic acid. The fed strain of *Pseudomonas aeruginosa* was identified as colonies which grew on Pseudomonas base agar with C-N supplement and could be agglutinated by serogroup-14 antiserum.

For enumeration of colonies of resistant *Enterobacteriaceae*, aliquots of stool were plated on MacConkey agar containing the antibiotic being administered in a concentration of 32 μg/ml for aztreonam or cefoxitin, or 64 μg/ml for cefoperazone or piperacillin. Colonies of resistant members of the *Bacteroides fragilis* group were counted on kanamycin-vancomycin laked blood agar plates containing antibiotic as described above. The emergence of resistant members of the normal flora was studied only in antibiotic-treated subjects and not in control subjects.

Results

Effects on Normal Fecal Flora

The effect of the four antibiotic regimens on the fecal microflora is summarized in Table 2. Cefoperazone had the most striking effect on *Enterobacteriaceae*, reducing the counts of these organisms to undetectable levels by the sixth day of treatment in all four subjects. Treatment with aztreonam caused a progressive decline in counts of *Enterobacteriaceae* to undetectable levels in three subjects; however, there was no appreciable change in the fourth subject. There was a modest overall reduction in these counts with piperacillin, but this was highly variable from subject to subject. Treatment with cefoxitin caused a transient increase in these counts in all four subjects.

Among the anaerobic flora, the predominant organisms were members of the *Bacteroides fragilis* group. Cefoperazone caused a reduction in counts to undetectable levels by the sixth day of treatment in all four subjects. Treatment with cefoxitin and aztreonam did not influence the counts of these bacteria. The effect of piperacillin was highly variable but was striking in only one subject in whom counts were reduced to undetectable levels. Cefoperazone caused a reduction in enterococci to undetectable levels in all four subjects. Treatment with piperacillin produced a variable effect. Administration of cefoxitin and aztreonam was associated with a consistent increase in enterococcal counts ranging as high as 6 to 7 logs.

The drug with the greatest impact on the fecal counts of yeasts was cefoperazone; all four volunteers had striking increases in counts, ranging from 5.8 to 7.3 logs. Three of four subjects in the cefoxitin and piperacillin groups had appreciable counts of yeasts in

Table 2: Effect of antibiotic regimen on composition of the fecal microflora.

Regimen	Drug concentration	*Enterobacteriaceae*	Anaerobes	Enterococci	Yeasts
Cefoxitin	low	↑	0	↑↑	↑
Aztreonam	low	↓↓	0	↑	0
Piperacillin	low	↓↓	↓	↓	↑↑
Cefoperazone	high	↓↓↓	↓↓↓	↓↓↓	↑↑↑

0 = maximum change of 0–2 logs; ↑ or ↓ = maximum change of 2–4 logs; ↑↑ or ↓↓ = maximum change of 4–6 logs; ↑↑↑ or ↓↓↓ = maximum change > 6 logs.

the stool. Only one subject treated with aztreonam had a detectable number of yeasts in the feces. Most of the fungal isolates were *Candida albicans*; however, *Torulopsis glabrata* and *Candida tropicalis* were recovered in a few instances. There was a significant ($p < 0.01$) inverse correlation between the log of the maximum increase in yeasts and the log of maximum decrease in anaerobes.

Effects on Colonization Resistance

In antibiotic-treated volunteers, the fed strains were detectable in the feces of only a few subjects after the first feeding and invariably had disappeared by the time of the second feeding. Therefore, the duration of elimination was measured from the time of the second feeding. However, control subjects, who received no antibiotic, were given only one feeding of bacteria and the duration of elimination was measured from the time of this single feeding. As shown in Table 3, the antibiotics had no apparent influence on the ability of the fed strains of *Escherichia coli* and *Pseudomonas aeruginosa* to colonize the intestinal tract; the *Escherichia coli* were detectable in the stools of 14 of 16 antibiotic-treated subjects and all four of the controls; the duration of elimination was brief, generally 3 to 5 days but exceeded one week in one instance. Similarly the *Pseudomonas aeruginosa* strain was found in the stools of 12 of 16 subjects receiving an antibiotic and three of four controls; the duration of elimination was quite variable but exceeded one week in six instances. For neither species could the duration of elimination be related to the antibiotic regimen which had been given or to the changes in the normal fecal flora.

Table 3: Colonization and duration of excretion of administered bacteria.[a]

	Escherichia coli	*Pseudomonas aeruginosa*
Cefoperazone	3/4 (5–13)	2/4 (5)
Cefoxitin	4/4 (3– 5)	4/4 (2–45)
Piperacillin	3/4 (3– 6)	2/4 (15–62)
Aztroenam	4/4 (3)	4/4 (3–60)
Controls	4/4 (3– 7)	3/4 (3–24)

[a]Number of subjects colonized/total subjects (range of colonization, days).

Table 4: Emergence of resistant strains during treatment.[a]

	Resistant *Enterobacteriaceae*	Resistant *Bacteriodes*
Cefoperazone	0/4	0/4
Cefoxitin	4/4 (0.2–10.5)	3/4 (8.8–11.4)
Piperacillin	1/4 (10.0)	2/4 (6.9–10.5)
Aztreonam	0/4	N.T.

[a]Number of subjects with resistant organisms/total subjects (range of counts of resistant organisms, log $_{10}$ per gram of feces).
N.T. = not tested.

Development of Antibiotic Resistance

The number of antibiotic-resistant colonies in the stool microflora detected during treatment with each drug is shown in Table 4. All four subjects in the cefoxitin group and one in the piperacillin group had remarkable increases in the numbers of resistant *Enterobacteriaceae* during treatment, whereas there were no such changes in the cefoperazone and aztreonam groups. Three subjects treated with piperacillin had a substantial increase in the number of resistant *Bacterioides* during treatment.

Colonization by other gram-negative bacilli, presumably acquired inadvertently from the environment, was also observed. One subject in the cefoperazone group, one in the piperacillin group, two in the aztreonam group, and three in the cefoxitin group were colonized by exogenous gram-negative rods, most often strains of *Pseudomonas aeruginosa*, during treatment (data not shown).

Discussion

Based on studies in animals, it has been stated that the anaerobic component of the intestinal microflora is essential to the maintenance of colonization resistance (11–14). Despite the lack of verification of these findings in humans, physicians are sometimes reluctant to use broad-spectrum antibiotics which may disturb the fecal flora, especially the anaerobic component. It has been recommended, for example, that antibiotic prophylaxis in leukemia patients be chosen with a view to sparing the anaerobic component of the fecal microflora (8, 22, 23). Thus, physicians are making antibiotic decisions based upon the unproved assumption that colonization resistance in humans is related to the anaerobic microflora.

In our studies we scrutinized the validity of the assumptions concerning anaerobes and colonization resistance. We assessed the microflora in antibiotic-treated volunteers in terms of the readiness with which gram-negative bacilli, either deliberately fed or randomly acquired from the environment, became implanted in the feces. Healthy volunteers were studied rather than sick patients in order to minimize the number of variables and because it would have been ethically difficult to feed potentially infective bacterial strains to sick persons (24). Our findings failed to show any evident relation between the antibiotic administered and the duration of excretion of the fed organisms. Failure to substantiate the concept of colonization resistance with these fed strains does not, of course, prove that concept is incorrect. We may have studied too [illegible] subjects or may have inadvertently chosen bacterial strains with poor colonizing properties.

While there was no correlation between intestinal colonization and antibiotic treatment with the fed strains, there were differences in random acquisition of gram-negative bacilli from the environment. Seven of sixteen antibiotic-treated volunteers but none of four control subjects became colonized by new gram-negative bacilli. Thus, we could demonstrate the phenomenon of colonization resistance in humans and showed that this protective mechanism is reduced by antibiotic administration.

The relation between colonization resistance and suppressive effects of the drug on various components of the fecal microflora was contrary to what some investigators have observed in animal studies (11–18). In our studies, for example, the cefoxitin-treated subjects, who showed minimal derangement in their aerobic and anaerobic microflora, were among the most heavily colonized, whereas the cefoperazone-treated subjects, who experienced the greatest alterations in their microflora, exhibited no discernible colonization by new microorganisms. Furthermore, subjects given cefoxitin or aztreonam, drugs that had the least effect on the anaerobic flora, experienced the greatest frequency of colonization by gram-negative bacilli. These findings argue against the concept that colonization resistance is related to maintenance of the anaerobic microflora of the intestinal tract (11–14).

The results of this study support the concept of colonization resistance operating in the intestine of humans, that is, the bowel flora is able to defend itself against invasion by exogenous microorganisms. It is also apparent that colonization resistance is impaired by antibiotic treatment, but the explanation is not understood. Although it may be unsettling to contemplate the deficits in our knowledge about colonization resistance, admission of our ignorance may stimulate research efforts to discover the real explanation for this important host defense mechanism.

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