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Antagonism Among the Normal Anaerobic Bacteria of the Mouse Gastrointestinal Tract Determined by Immunofluorescence

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Strictly anaerobic *Bacteroides* sp., *Eubacterium* sp., and *Fusobacterium* sp. were isolated from the cecum of a conventional mouse. An immunofluorescent method utilizing rabbit antisera specific for each of these three strains was developed to determine their population levels in the gastrointestinal tracts of gnotobiotic mice. Population levels of these anaerobes in groups of gnotobiotic mice colonized with either *Bacteroides, Eubacterium,* or *Fusobacterium* were compared with those of gnotobiotes colonized with all three strains. *Bacteroides* population levels in gnotobiotes colonized with all three strains were 100-fold less than the *Bacteroides* population level in gnotobiotes colonized with only the *Bacteroides* strain. *Eubacterium* or *Fusobacterium* population levels were not reduced by the presence of the other anaerobic strains. Thus, strictly anaerobic *Eubacterium* sp. and *Fusobacterium* sp. that colonized gnotobiotic mice caused a reduction in the in vivo population levels of a strictly anaerobic *Bacteroides* sp.

Special relationships exist between host animals and their indigenous gastrointestinal microflora. Studies with germfree animals demonstrate that these indigenous bacteria profoundly influence the anatomical, physiological, and immunological characteristics of the host (13). Conversely, the composition and population levels of the indigenous microbes themselves are influenced by interactions with each other and with the host (10). Little is known concerning the actual mechanisms functioning in vivo in these complex ecological interrelationships among the various members of the gastrointestinal flora or between the flora and the host.

The composition and population levels of the indigenous bacteria colonizing the gastrointestinal tract probably are influenced or controlled by many different mechanisms. Two basic mechanisms that may play important roles in controlling the flora are: (i) local immunity (secretory immunoglobulin A) to antigens of the indigenous bacteria and (ii) bacterial antagonism among the various members of the flora. Any study attempting to elucidate the mechanisms that control the composition and population levels of the indigenous gastrointestinal bacteria should include the strictly anaerobic bacteria, since they are the predominant members of the flora (6). There is no direct evidence that the immune response of the host has any influence on the populations of the strictly anaerobic bacteria indigenous to the mouse gastrointestinal tract. In fact, it is difficult to elicit a strong immune response by mice to antigens of these indigenous anaerobes (3, 4).

Bacterial antagonism, sometimes called bacterial interference, is defined as the inhibition of growth or the reduction in numbers of one bacterial species by one or more other bacterial species. The extreme complexity of the indigenous bacterial flora of conventional mice makes it very difficult to design experiments that demonstrate conclusively the mechanisms operating in vivo to control the composition and population levels of certain indigenous bacteria. The many members of the indigenous flora interact with each other and with the host to introduce additional environmental effects on the particular bacterial population being examined. Germfree mice, however, are free from a diverse microflora and can be colonized with a simplified bacterial flora of the particular bacterial species to be examined. This paper describes the development of an in vivo experimental model of bacterial antagonism among three strictly anaerobic bacteria of different genera in the gastrointestinal tracts of gnotobiotic mice.

MATERIALS AND METHODS

Animals. The initial breeding stock of germfree, CD-1 mice was purchased from Charles River Breeding Laboratories, Wilmington, Mass. The experimen-

tal germfree and gnotobiotic mice were housed in sterile stainless-steel cages with suspended, wire mesh bottoms inside Trexler-type flexible vinyl isolators previously sterilized with 2% peracetic acid (FMC, Buffalo, N.Y.). Sterility tests were performed by established procedures (28).

The mice were fed Teklad autoclavable diet (L-485); (Teklad Mills, ARS/Sprague-Dawley, Winfield, Iowa) autoclaved at 16 lb/in^2 for 25 min with a 28-inch (71.1 cm) vacuum cycle 10 min before and after sterilization.

Adult New Zealand white rabbits were obtained from Langshaw Farms, Augusta, Mich., and were fed Teklad rabbit diet.

Bacteria. Streptomycin-resistant *Escherichia coli* C25 (7) was grown at 37°C on deoxycholate lactose agar plates (Difco Laboratories, Detroit, Mich.) containing 1 mg of streptomycin sulfate (Pfizer Inc., New York, N.Y.) per ml. The *Bacteroides* sp., *Fusobacterium* sp., and *Eubacterium* sp. were kindly furnished by Rolf Freter of the University of Michigan, Ann Arbor. These three strains of anaerobes were isolated from the cecum of a single conventional BALB/wm mouse at 37°C in an anaerobic glove box (Coy Laboratory Products, Inc., Ann Arbor, Mich.) on enriched Trypticase soy agar (TSA; Baltimore Biological Laboratories, Cockeysville, Md.) containing palladium chloride (0.33 g/liter) (1). The growth medium was prereduced for 48 h in the anaerobic glove box before use. The atmosphere inside the glove box was monitored daily for oxygen contamination with a trace oxygen analyzer (Lockwood and McLorie, Inc., Horsham, Pa.) adapted to measure oxygen in this special contained atmosphere of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen. Oxygen contamination inside the glove box was maintained at less than 10 parts of oxygen per 10^6 parts of atmosphere throughout all experiments. These strains of strictly anaerobic bacteria were identified by W. E. C. Moore at the Virginia Polytechnic Institute (VPI) Anaerobic Laboratory, Blacksburg. They did not fit into any of the various taxonomic schemes and, therefore, could only be classified at the genus level. They are members of the collection of F strains described by Syed et al. (25) and Freter and Abrams (11). The number designations given by Freter and by VPI are: *Bacteroides* sp., F1, VPI 7735; *Eubacterium* sp., F53, VPI 7977; and *Fusobacterium* sp., F102, VPI 7965. These particular bacterial strains were chosen because they occur in high population levels in the mouse gastrointestinal tract, and rabbits injected with them have produced high-titer antisera.

Colonization of germfree mice with *E. coli* C25 and the strictly anaerobic bacteria. Syed et al. (25) previously demonstrated that to colonize germfree mice with certain strict anaerobes it is first necessary to colonize the mice with *E. coli* C25 or other suitable strains to lower the oxidation-reduction potential in their gastrointestinal tracts. *E. coli* C25 was cultured at 37°C on deoxycholate lactose agar plates containing 1 mg of streptomycin sulfate per ml. The *E. coli* C25 cells were suspended in Trypticase soy broth (TSB) and sealed inside autoclaved glass ampoules. The outsides of the sealed ampoules were sterilized with 2% peracetic acid and introduced into the germfree isolators. The *E. coli* C25 cultures were suspended in the drinking water of the mice. Colonization of the inoculated mice was confirmed by culturing the mouse feces.

Bacteroides, Eubacterium, and *Fusobacterium* strains were cultured on enriched Trypticase soy agar plates containing 0.033% palladium chloride at 37°C in an anaerobic glove box. These anaerobes did not grow well in enriched TSB. All media were prereduced in the anaerobic glove box at least 48 h before use. The three strains of anaerobes were harvested after various incubation periods, since they grew at different rates on the agar plates. The greatest numbers of viable *Bacteroides, Eubacterium,* and *Fusobacterium* anaerobes were harvested after 48, 24, and 36 h of incubation, respectively.

The inoculation medium consisted of TSB containing 0.3 M phosphate buffer (pH 7.5) to neutralize the stomach acidity of the germfree mice and a final concentration of 0.05% dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) to reduce any oxygen contamination during the intragastric inoculations. Homogenates of germfree mouse stomachs were titrated in vitro with 0.3 M phosphate buffer (pH 7.5) to determine the concentration needed to neutralize stomach acidity. Germfree mice also were intragastrically inoculated with various concentrations of dithiothreitol to determine its toxicity levels. Intragastric inoculations of 0.05% dithiothreitol into germfree mice caused no apparent reactions in these mice over a 30-day period. Higher concentrations, however, were rapidly fatal to these mice. The TSB inoculation medium containing 0.3 M phosphate buffer and 0.05% dithiothreitol was sterilized by filtration with 0.22-μm disks (Millipore Corp., Bedford, Mass.) and prereduced in an anaerobic glove box for 48 h.

Each of the strictly anaerobic strains was grown on enriched TSA plates in an anaerobic glove box for the optimal incubation time as described above. The bacterial growth was scraped off the plates and suspended in the TSB mixture. The number of bacteria of each anaerobic strain was determined by viable plate counts on enriched TSA. The desired concentrations of bacteria were transferred to sterile glass tubes in the anaerobic glove box and then stoppered with sterile, soft rubber stoppers. The outsides of the tubes containing the strict anaerobes were then sterilized with 2% peracetic acid and introduced into germfree isolators. Inside the germfree isolators, a beveled 2.5-inch (6.5-cm), 22-gauge disposable needle and a 2.5-ml glass syringe were used to penetrate the rubber stopper and withdraw the bacterial inocula. This needle then was replaced with a 2.5-inch, 22-gauge stainless-steel feeding needle with a stainless-steel bulb on the tip (Popper and Sons, Inc., New Hyde Park, N.Y.) for inoculating the mice. The inoculum was injected into each mouse intragastrically and rectally, and the addition of dithiothreitol to it should have reduced any oxygen contamination at this stage of the inoculation procedure. Colonization of the mice was confirmed by examining fecal smears stained with fluorescein-labeled antisera specific for each of the anaerobic strains in the inocula.

Determination of viable bacterial population levels in the gastrointestinal tracts of gnotobiotic mice by viable plate counts. Gnotobiotic mice were removed from the germfree isolators, killed

by cervical dislocation, and then placed in an anaerobic glove box. Various gastrointestinal organs were removed from the mice by sterile procedures, weighed, and placed in separate, sterile homogenizing jars (VirTis Research Equipment Co., Gardiner, N.Y.) containing known volumes of prereduced TSB. The organs were homogenized at medium speed for 10 s with the VirTis homogenizer in the anaerobic glove box.

Serial dilutions of the organ homogenates were prepared in prereduced TSB. Strict anaerobes were cultured selectively by spreading the dilutions with glass L-rods onto enriched TSA plates containing 0.033% palladium chloride and 100 U of polymyxin B sulfate per ml to inhibit growth of *E. coli* C25. The anaerobic bacteria were not inhibited by this concentration of polymyxin B. Selective deoxycholate lactose agar plates containing 1.25 g of streptomycin sulfate per liter were used to culture *E. coli* C25. The deoxycholate lactose agar plates were incubated at 37°C aerobically for 24 h and the enriched TSA plates for 48 to 60 h at 37°C in the anaerobic glove box. The numbers of viable bacteria were computed per gram of tissue.

Preparation of fluorescein-conjugated antisera to the bacterial strains. Antisera were harvested from rabbits after intravenous injections of live bacterial cells of each strain suspended in normal saline. The bacteria were grown on agar plates and then a heavy bacterial suspension was prepared in normal saline. Four injections of 0.2, 0.4, 0.8, and 1.6 ml each were given at 5-day intervals, followed 1 week later by the first of 3 weekly 3.2-ml injections. Rabbits were bled 5 days after each series of immunizations. Each antiserum was precipitated with 35% saturated ammonium sulfate by the procedure of Hebert et al. (14). The precipitate was suspended in distilled water equal to 45% of the original volume of serum and dialyzed overnight against 0.01 M sodium phosphate buffer in 0.85% NaCl (pH 7.3).

The protein concentration of the precipitated antiserum was determined spectrophotometrically by the procedure of Waddell and Hill (27). Carbonate-bicarbonate buffer (pH 9.0) was added to make a 10.0% final concentration. Fluorescein isothiocyanate isomer I (Baltimore Biological Laboratories) was then added slowly, and the mixture was stirred slowly at 4°C overnight (0.025 mg of fluorescein per mg of protein). The fluorescein-conjugated antiserum was passed through a column of Sephadex G-25 with phosphate-buffered saline (pH 7.3) elution buffer to remove the unconjugated dye. The labeled-antiserum eluate was dialyzed at 4°C overnight against phosphate-buffered saline (pH 7.3). Each labeled antiserum was tested for specificity by the direct immunofluorescence test by using suspensions of each bacterial species cultured in vitro. The only cross-reactivity detected was between antisera to *E. coli* C25 and *Fusobacterium* antigens. Consequently, these antisera were absorbed with the cross-reacting bacteria until specificity was achieved.

Determination of the bacterial population levels in the gastrointestinal tracts of gnotobiotic mice by immunofluorescence methods. The intestinal organs to be quantitated for bacterial population levels were weighed and then homogenized using sterile procedures inside the anaerobic glove box as described above. Samples of the homogenates were plated on enriched TSA containing polymyxin and on deoxycholate lactose agar containing streptomycin to obtain viable bacterial plate counts for comparison with the bacterial counts obtained by the immunofluorescence test. The organ homogenates then were removed from the anaerobic glove box. A homogenate suspension (0.2 ml) was mixed 1:1 in 5-ml Corex round-bottom centrifuge tubes with fluorescein-labeled antiserum specific for a particular bacterial species. The mixture of organ homogenate and fluorescein-conjugated antiserum was allowed to react for 30 min at room temperature. The stained homogenate then was washed twice with phosphate-buffered saline (pH 7.3) by centrifugation at 39,100 × *g* at 4°C. The pellet obtained was resuspended with phosphate-buffered saline (pH 7.3) to a final concentration that allowed accurate counting of the bacteria. Fluorescein-stained bacteria were then counted in Petroff-Hausser counting chambers with a Zeiss fluorescence microscope with a UG1 exciter filter and a quartz-halogen lamp (Carl Zeiss, Inc., New York, N.Y.). The bacteria in the counting chambers were also counted by dark-field illumination. The number of bacteria was computed per gram of tissue. Tissue homogenates were reacted with normal rabbit sera (precipitated with 35% ammonium sulfate) that were conjugated with fluorescein as negative controls. The number of fluorescing bacteria was compared against the number of bacteria counted by dark-field illumination which served as a control.

RESULTS

Colonization of germfree mice with strictly anaerobic bacteria. A great deal of difficulty was encountered in colonizing germfree mice with the strictly anaerobic *Bacteroides, Eubacterium,* and *Fusobacterium* strains. Colonization was achieved by adopting the following procedures: (i) colonizing the germfree mice with *E. coli* C25 for 1 week to lower the oxidation-reduction potential in the gastrointestinal tract before inoculation with the strict anaerobes, (ii) preparing the inocula with anaerobes that had been incubated for the particular time period on agar plates yielding the greatest number of viable cells of each strain (48 h for *Bacteroides,* 24 h for *Eubacterium,* and 36 h for *Fusobacterium*), (iii) suspending the anaerobes in TSB containing 0.3 M phosphate buffer to neutralize stomach acidity and 0.05% dithiothreitol to reduce any oxygen contamination, (iv) transferring the bacterial inocula from the anaerobic glove box to the germfree isolators in tightly stoppered glass tubes, and (v) inoculating the mice intragastrically and rectally with the anaerobic strains to reduce oxygen contamination.

With the above procedures, only 50% of the germfree mice were colonized by the *Bacteroides* strain after each intragastric inoculation,

whereas all of the germfree mice were colonized by the *Eubacterium* or *Fusobacterium* strains after the inoculations. Furthermore, germfree mice housed in cages with gnotobiotic mice colonized with *E. coli* C25 and *Bacteroides* did not become colonized "naturally" with *Bacteroides. Eubacterium* and *Fusobacterium,* however, readily colonized germfree mice placed in cages with gnotobiotic mice that were already colonized with these organisms.

Determination of the population levels of the strictly anaerobic bacteria in gnotobiotic mice. Immunofluorescent counts of bacteria are usually performed by staining smears of the bacteria on glass slides followed by several washings with buffered saline. It is likely that some of the bacteria are washed off the slides during these washing procedures. It is also possible that the degree of "adhesion" of the bacteria to the glass slides is dependent upon the characteristics of each bacterial species. The various strains of strict anaerobes might adhere to the glass slides to different degrees, thereby causing errors in the quantitation of each strain. Consequently, homogenates of intestinal tissue from gnotobiotic mice colonized with either *Bacteroides, Eubacterium,* or *Fusobacterium* were stained with specific antisera conjugated with fluorescein, and the fluorescing bacteria were counted in liquid suspension in Petroff-Hausser counting chambers. The population levels of each of the anaerobic strains in the ceca of monoassociated gnotobiotic mice were compared by viable bacterial plate counts, immunofluorescent bacterial counts, and bacterial counts utilizing dark-field illumination (Table 1). Anaerobic bacteria are quite large and distinctive in shape, and they were easily counted by using dark-field illumination in homogenates from these monoassociated gnotobiotes. Approximately 100 times more anaerobes were detected by the immunofluorescent method than by viable plate counts. All of the bacteria present in the ceca (both living and dead) were counted accurately by the immunofluorescence procedure, since nearly identical numbers of bacteria were counted with either the fluorescence or dark-field method. Thus, the strains of strictly anaerobic bacteria could be quantitated accurately by the immunofluorescent counting method.

Demonstration of antagonism among strictly anaerobic bacteria in the gastrointestinal tracts of gnotobiotic mice. Germfree mice were colonized for 1 week with *E. coli* C25 and then divided into four groups and housed in separate isolators. Each of three groups of these monoassociated gnotobiotic mice was inoculated intragastrically and intrarectally with either *Bacteroides, Eubacterium,* or *Fusobacterium* strains. The fourth group of monoassociated mice was inoculated with all three strains of the strict anaerobes. The population levels of each of the anaerobic strains in the ceca of these mice were compared after 3 and 7 weeks of colonization (Table 2). *Bacteroides* population levels determined by immunofluorescent counts were approximately 100-fold less in gnotobiotes colonized with all three anaerobic strains plus *E. coli* C25 as compared with their levels in gnotobiotes colonized with only *Bacteroides* and *E. coli* C25. *Eubacterium* population levels, however, were not reduced in the presence of *Bacteroides* and *Fusobacterium. Eubacterium* or *Fusobacterium* population levels were not reduced even after association with the other anaerobic strains for

TABLE 1. *Comparison of methods for determining the population levels of each strain of anaerobe colonizing the ceca of gnotobiotic mice*

Gnotobiotes* colonized with	Viable** plate counts	Immuno-*** fluorescent counts	Darkfield illumination counts
Bacteroides	7.0**** (6.5–7.5)	9.1 (8.6–9.7)	9.5 (9.4–9.6)
Eubacterium	8.3 (7.3–9.2)	10.0 (9.7–10.2)	10.6 (10.4–10.7)
Fusobacterium	7.2 (6.4–8.8)	10.6 (10.4–10.8)	10.6 (10.4–10.8)

* Germfree CD-1 mice monoassociated with *E. coli* C25 1 week before intragastric inoculation with the strict anaerobes; five mice per test.

** Viable plate counts on enriched TSA containing palladium chloride.

*** Direct immunofluorescent counts with rabbit antisera conjugated with fluorescein.

**** Mean $\log_{10}$ numbers of bacteria per gram of cecum; ranges in parentheses.

TABLE 2. *Antagonism among the strictly anaerobic bacteria colonizing the ceca of gnotobiotic mice*

Gnotobiotes colonized with *E. coli* C25 plus*	Bacterial strain counted	Time after inoculation with anaerobes	
		3 weeks	7 weeks
Bacteroides	*Bacteroides*	10.1** (10.0–10.2)	10.2 (10.0–10.3)
Bacteroides *Eubacterium* *Fusobacterium*	*Bacteroides*	8.4 (8.0–8.7)	8.3 (8.0–8.6)
Eubacterium	*Eubacterium*	9.9 (9.6–10.1)	9.8 (9.4–10.0)
Bacteroides *Eubacterium* *Fusobacterium*	*Eubacterium*	9.9 (9.7–10.0)	9.7 (9.6–9.8)
Bacteroides	*E. coli* C25	10.5*** (10.3–10.7)	8.2 (7.9–8.4)
Eubacterium	*E. coli* C25	10.5 (10.2–10.8)	9.2 (8.2–9.5)
Bacteroides *Eubacterium* *Fusobacterium*	*E. coli* C25	10.2 (10.0–10.3)	8.5 (8.2–8.6)

* Gnotobiotic mice colonized with *E. coli* C25 1 week before inoculation with the anaerobic strains.

** Mean $\log_{10}$ immunofluorescent bacterial counts per gram of cecum, utilizing specific rabbit antisera conjugated with fluorescein. Ranges in parentheses; five mice per test.

*** Mean $\log_{10}$ viable plate counts on deoxycholate lactose agar containing 1 mg of streptomycin per ml.

30 weeks in gnotobiotic mice (Table 3). Thus, strictly anaerobic *Eubacterium* and *Fusobacterium* strains colonizing the ceca of gnotobiotic mice caused a reduction in the in vivo population levels of a strictly anaerobic *Bacteroides* strain. This in vivo model demonstrates antagonism among strictly anaerobic bacteria indigenous to the mouse gastrointestinal tract.

DISCUSSION

The *Bacteroides* sp., *Eubacterium* sp., and *Fusobacterium* sp. are not distinguishable by their colonial morphology on agar plates. Thus, the viable plate count method could not be used for quantitating bacterial antagonism among these anaerobes. Furthermore, the bacterial strains not being antagonized would overgrow the agar plates spread with the dilution of cecal homogenate and the antagonized strain could not be quantitated. In preliminary tests, these three anaerobic bacteria also could not be separated by being cultured on agar media containing various antibiotics, dyes, or other inhibitory chemicals. It was possible, however, to determine the population level of each anaerobic strain in the mixed flora of the gnotobiotes by counting only the bacterial strain that fluoresced after reacting with fluorescein-conjugated antisera specific for each strain. Both living and dead bacteria can be counted by this immunofluorescence method. Immunofluorescence counts, however, may provide more reliable data than viable plate counts, since the anaerobes unable to grow on the enriched TSA plates will still be counted by the immunofluorescence method. It is possible that a particular strict anaerobe will grow on the enriched TSA only if it has reached a certain physiological state or growth stage at the time of culture. The physiological state of the bacteria, however, should not influence the immunofluorescent counts.

Bacterial antagonism or interference among various bacterial species has been demonstrated on the skin (23) and in the mouth (26), throat (24), and intestines (8). There are several reports of in vitro and in vivo bacterial antagonism of aerobic and facultatively anaerobic bacteria by the strictly anaerobic bacteria. *Bacteroides fra-*

TABLE 3. *Lack of antagonism of Eubacterium and Fusobacterium during long-term colonization*

Gnotobiotes colonized for 30 wks with *E. coli* C25 plus	Anaerobic strain counted	Cecum	Large intestine
Eubacterium	*Eubacterium*	9.3* (9.0–9.5)	9.2 (9.2–9.2)
Bacteroides *Eubacterium* *Fusobacterium*	*Eubacterium*	9.4 (9.2–9.5)	8.6 (8.5–8.8)
Fusobacterium	*Fusobacterium*	9.9 (9.7–10.1)	9.6 (9.5–9.8)
Bacteroides *Eubacterium* *Fusobacterium*	*Fusobacterium*	9.3 (9.1–9.6)	8.9 (8.7–9.3)

* Mean $\log_{10}$ numbers of bacteria per gram of cecum determined by immunofluorescent counts utilizing specific rabbit antisera conjugated with fluorescein. Ranges in parentheses; three mice per test.

gilis inhibits the in vitro growth of *Salmonella enteritidis* (5), *Shigella flexneri* (15), and *Pseudomonas aeruginosa* (17). *B. melaninogenicus* from the human oropharynx also inhibits the in vitro growth of certain indicator strains of facultative *Enterobacteriaceae* (19). Lee and Gemmell (16) suggested that certain volatile fatty acids (especially butyric acid) produced by fusiform-shaped, anaerobic bacteria reduce the population levels of coliforms in mouse gastrointestinal tracts. Orcutt and Schaedler (20) reported that an anaerobic, fusiform-shaped bacterium inhibits the growth of *Staphylococcus aureus* in gnotobiotic mice colonized with both of these bacterial types. Maier and Hentges (18), however, found that *B. fragilis* does not reduce the growth of *S. flexneri* in gnotobiotic mice. In my study, population levels of *E. coli* C25 in gnotobiotic mice were not reduced significantly by *Bacteroides, Fusobacterium,* and *Eubacterium* (Table 2). *E. coli* C25 maintained similar population levels in gnotobiotes monoassociated with *E. coli* C25. These results are in agreement with earlier experiments in continuous-flow cultures (12) and in gnotobiotic mice (25) which demonstrated that *E. coli* C25 population levels are antagonized by the entire cecal flora but not by only a few strains of strict anaerobes. Thus, certain strict anaerobes of the human or mouse flora apparently can limit the in vitro and in vivo growth of particular aerobic and facultatively anaerobic bacteria, but only under certain environmental conditions.

The experiments described in this paper demonstrate that the population levels of a strictly anaerobic strain of *Bacteroides* in the ceca of gnotobiotic mice were reduced approximately 100-fold by the presence of strictly anaerobic strains of *Eubacterium* and *Fusobacterium.* Raibaud and Ducluzeau (21) have also observed bacterial antagonism among strictly anaerobic bacteria in gnotobiotic mice. To my knowledge, these are the only reports of bacterial antagonism operating among the strictly anaerobic bacteria that comprise the normal gastrointestinal flora.

The mechanism(s) whereby *Eubacterium* and/or *Fusobacterium* reduce the population levels of *Bacteroides* in gnotobiotes is unknown. Speculations have been reported concerning the mechanisms of antagonism of facultatively anaerobic bacteria by the strict anaerobes. Baskett and Hentges (2) suggest that the anaerobes produce volatile fatty acids, such as acetic and butyric acids, which inhibit the metabolism of *Shigella* cells. These organic acids apparently penetrate the bacterial cell membrane at low pH and produce their toxic effects intracellularly. Levison (17) speculates that fatty acids may uncouple oxidative phosphorylation and inhibit adenosine triphosphate-inorganic PO_4 exchange. I know of no studies, however, concerning the mechanism(s) whereby strictly anaerobic bacteria reduce the population levels of other strict anaerobes. The mechanisms described above for bacterial antagonism of facultatively anaerobic bacteria by the strict anaerobes may or may not apply in the case of bacterial antagonism among

the strictly anaerobic bacteria themselves. It seems likely, however, that these strict anaerobes would compete for energy sources and particular environmental niches in the gastrointestinal tracts of mice.

The mechanisms operating during bacterial antagonism are dependent upon existing environmental conditions. Freter (9, 10) suggests that bacterial antagonism of a population of *E. coli* C25 operates by at least three mechanisms in anaerobic continuous flow cultures consisting of the entire cecal microflora of a mouse plus *E. coli* C25. The three mechanisms are: (i) competition for carbon and energy sources, (ii) inhibitory fatty acids functioning at low pH, and (iii) an unidentified volatile inhibitor. The environment in the continuous flow culture determines which mechanism(s) is operating at any particular time. Thus, the environment selects for the mechanism(s) operating in bacterial antagonism and complicates attempts to elucidate these mechanisms in vivo. These studies are further complicated because the physiological state of the population of bacterial species being examined may determine whether or not this organism antagonizes or is antagonized by other bacteria. For example, a bacterium that is already adapted to the environment of the gastrointestinal tract is less likely to be inhibited by bacterial antagonism than one that has not adapted previously to this environment (22). Therefore, the elucidation of the mechanisms by which bacterial antagonism operates in vivo will be very difficult because of the complex interactions between the environment and these mechanisms.

The results described in this paper demonstrate that bacterial antagonism can occur in vivo between certain strictly anaerobic bacteria that normally colonize the mouse gastrointestinal tract. This gnotobiotic mouse model of bacteria antagonism among strictly anaerobic bacteria may prove useful for identifying the actual mechanisms operating during bacterial antagonism. This model can also be used to test other hypotheses concerning mechanisms whereby the host controls the composition and population levels of its indigenous microflora. For example, Shedlofsky and Freter (22) found that local immunity and bacterial antagonism act synergistically to reduce the population levels of *Vibrio cholerae* in the gastrointestinal tracts of gnotobiotic mice. Local immunity against *V. cholerae* was more effective in reducing the vibrio population size in vivo when the numbers of vibrios had been lowered previously by the antagonistic action of *Proteus vulgaris, Enterobacter aerogenes, Streptococcus faecalis,* and two strains of *E. coli.* Possibly, local immunity and bacterial antagonism also operate synergistically to control the population levels of certain strict anaerobes indigenous to the mouse gastrointestinal tract. This hypothesis can be tested by utilizing the gnotobiotic model of bacterial antagonism. Information obtained with this gnotobiotic mouse model can then be used to design experiments to identify the mechanisms operating during bacterial antagonism in the complex bacterial flora of conventional mice.

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