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Inhibition of *Shigella flexneri* by the Normal Intestinal Flora

III. Interactions with *Bacteroides fragilis* Strains in Vitro

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Five strains of *Bacteroides fragilis* isolated from human feces were assayed for their inhibitory activities against *Shigella flexneri* in vitro. Inhibition was not detected when *Shigella* and the *Bacteroides* strains were simultaneously inoculated into a defined liquid medium containing glucose. Inhibition was apparent only when *Shigella* was inoculated into established *Bacteroides* cultures. In exponential-phase cultures, the *Shigella* growth rate was diminished and logarithmic growth was prematurely interrupted. *Shigella* failed to multiply at all in stationary-phase *Bacteroides* cultures. An analysis of the stationary-phase cultures revealed that acetic and propionic acids were present in sufficiently high concentrations and the *p*H of the culture medium was sufficiently low to account for the inhibition of *Shigella* growth. In glucose-free *Bacteroides* cultures, *Shigella* multiplied almost as well as in control cultures, despite the presence of high concentrations of volatile fatty acids. At the high *p*H levels present in the glucose-free cultures, the acids were not toxic for *Shigella*. These results reaffirm the importance of the relationship between volatile fatty acids and *p*H in the inhibition of *Shigella* growth.

In assaying the inhibitory activity of intestinal bacteria against *Shigella flexneri*, we recently demonstrated that coliform organisms inhibit *Shigella* growth in mixed cultures (7, 9). Inhibition is due to the production of formic and acetic acids by the coliform strains. Under the reducing conditions and at the *p*H levels attained in the cultures, the volatile fatty acids exert a bactericidal effect on *Shigella*.

Although coliform organisms constitute an important segment of the indigenous intestinal flora, there is recent evidence that anaerobic bacteria, such as *Bacteroides*, are the predominant organisms in the intestines of man and many animals (5, 13, 14). Because of their predominance, we have undertaken an investigation of the inhibitory activities of several strains of *Bacteroides* against *Shigella*. This represents, to the best of our knowledge, the first attempt to study interactions between *Shigella* and intestinal anaerobes. In this paper, we report the results of experiments that explore the relationships between *Shigella* and *Bacteroides* and explain the mechanisms by which *Bacteroides* inhibits *Shigella* growth in vitro. Cultural conditions under which *Bacteroides* fails to inhibit *Shigella* growth in mixed culture are also described.

MATERIALS AND METHODS

Microorganisms. The *Shigella* strain, which was used in previous studies (7–10), was identified serologically as *S. flexneri* 2a. The *Bacteroides* strains were isolated from human feces. A number of tests, listed in Table 1, were used to identify these strains as *B. fragilis*. The strains were arbitrarily assigned numbers (1 through 5) for purposes of differentiation.

Anaerobic chamber. An anaerobic glove box isolator, described by Aranki et al. (1), was assembled for these studies. It consisted, essentially, of an entry lock and a large, flexible, clear, vinyl chamber with attached gloves. The chamber was filled with a mixture of 85% nitrogen, 10% hydrogen, and 5% CO_2, which was circulated over a palladium catalyst to eliminate trace oxygen. Inclusion of a heating element and thermoregulator for incubation (37 C) permitted processing of the cultures entirely within the chamber.

Media. Veal Infusion Agar (BBL) was used for plating *Shigella* cultures. A special medium, enriched Trypticase Soy Agar (ETSA), prepared as described by Aranki et al. (1), was used for plating *Bacteroides* cultures. ETSA plates were prepared outside the chamber with an overlay of palladium black. A drop of phenosafranine indicator was added to two control plates. After introduction into the anaerobic chamber, the ETSA plates were not used until the phenosafranine indicator in the control plates became colorless. Mixed-culture studies were carried out in a defined

TABLE 1. *Identification of Bacteroides*

Determination	Result
Cell morphology......	Gram negative bacilli, rounded ends, somewhat pleomorphic
Colony morphology...	Round, entire, smooth, gray
Motility..............	Negative
Acid from glycerol....	Negative
Gelatin hydrolysis....	Negative
Glucose fermentation	
Terminal *p*H........	4.91[a]
Volatile acids produced............	Acetic and propionic[b]
Growth stimulation, 10% bile..........	Positive
Growth in the presence of	
Polymyxin (10 μg/ml)...............	Positive
Penicillin (10 μg/ml)...............	Negative
Hydrogen sulfide production...........	Positive (slight)
Indole production....	Negative
Behavior in cysteine milk	
Terminal *p*H........	4.81[a]
Clot formation.....	Positive

[a] Average values for the five strains.
[b] Four of five strains produced small quantities of propionic acid.

liquid medium (DL medium) consisting of 1.7% Trypticase (BBL), 1.25% glucose, 0.5% yeast extract, 0.2% placenta powder, 0.5% NaCl, 0.65% Na_2HPO_4, 0.08% KH_2PO_4, 0.042% Na_2CO_3, 0.05% cysteine-HCl, 1 μg of hemin per ml, and 0.5 μg of menadione per ml. The *p*H of the medium was 6.82, without adjustment. For some experiments, as indicated in the text, glucose was either deleted from the medium or its concentration was reduced to 0.25%. After preparation, the medium, in 10-ml quantities, was placed in screw-cap tubes (18 by 150 mm). The tubes were stored in the anaerobic chamber for 48 hr before use, to allow for reduction of the medium.

Preparation of inocula. *Bacteroides* stock cultures were streaked on ETSA plates in the anaerobic chamber. The plates were placed in a plastic container fitted with a wire-mesh bottom. A slurry of $PbCO_3$ beneath the wire mesh absorbed H_2S produced by the organisms during growth. Isolated colonies that developed on the plates after 72 hr of incubation were picked to DL medium. The liquid cultures were incubated for 48 hr, after which serial dilutions were prepared from them in saline to give inocula of approximately 10^3 organisms per ml. *Shigella* stock cultures were streaked on Veal Infusion Agar plates. Colonies that developed on the plates after 24 hr of incubation were picked to DL medium. After exactly 18 hr of incubation, the *Shigella* cultures were introduced into the anaerobic chamber, and serial dilutions were prepared from them to give inocula of 10^3 to 10^5 organisms per ml.

Determination of population size in pure and mixed cultures. All liquid cultures were incubated at 37 C in the anaerobic chamber. At intervals during incubation, samples were removed from the cultures and dilutions were prepared from them in saline. Portions (1 ml) of the dilutions were flooded on the surface of either dried Veal Infusion Agar or ETSA plates (6). ETSA plates used for enumeration of *Bacteroides*, were incubated for 72 hr in the anaerobic chamber. Veal Infusion Agar plates, used for enumeration of *Shigella*, were removed from the chamber for incubation in an air incubator at 37 C for 24 hr. Colonies that developed on the surface of the agar plates were counted with an electronic colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Analyses of environmental changes in liquid cultures. Measurements of *p*H were made with a Beckman Expandomatic pH Meter, model 76, by use of a standard combination electrode (Beckman no. 39142). The electrode was immersed directly into the liquid cultures. Oxidation-reduction potential measurements in liquid cultures were made within the anaerobic chamber by use of a platinum combination electrode (Beckman no. 39186) which was connected by a lead to the *p*H meter located outside the chamber. For extraction of volatile acids, cultures were acidified to *p*H 1 with concentrated H_2SO_4 and were steam-distilled until four times the original volume of material was collected as distillate. After condensation of the distillate, the acids were separated on a Celite 545 column with 5% *n*-butyl alcohol in chloroform and were titrated with 0.05 N NaOH (12). Sodium ions were removed from the fractions by passage through an ion-exchange column (acid-washed Dowex 50, 30 to 50 mesh). Acids in the fractions were then identified by paper chromatography (4).

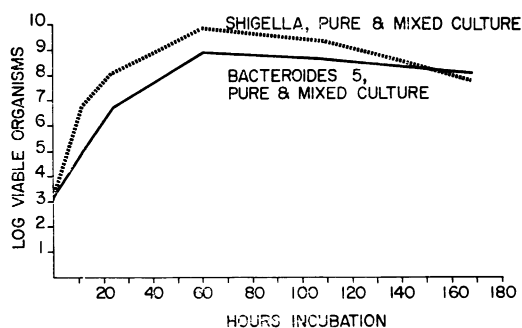

FIG. 1. *Growth curves for Shigella and Bacteroides 5 in DL medium under anaerobic conditions. The growth curves were identical for each organism when they were grown separately in pure culture and when they were grown together in mixed culture.*

RESULTS

Five strains of *B. fragilis* isolated from human feces were assayed for their inhibitory activities against *S. flexneri* in vitro. Results obtained with the five strains were nearly identical. For this reason, the experimental data are frequently illustrated by use of a representative *Bacteroides* strain, designated *Bacteroides* 5.

Typical pure- and mixed-culture growth curves for *Shigella* and *Bacteroides* are illustrated in Fig. 1. Because of the similarity of results obtained when pure- and mixed-culture curves for each organism were plotted, both curves, in each case, are represented by a single line. By comparing the slopes of the lines representing exponential growth of the organisms, it can be seen that the *Shigella* growth rate was greater than the growth rate of *Bacteroides* 5. Both organisms attained a maximal viable population at about 60 hr, at which time the *Shigella* population was approximately 10 times greater than the *Bacteroides* 5 population. The organisms then entered into an extended logarithmic death phase. It is apparent from these data that neither organism interfered with the multiplication of the other in the mixture. No inhibition could be detected.

Bacteroides is normally present, in large numbers, as part of the indigenous intestinal flora of man and a variety of animals. To simulate conditions existing during exposure to *Shigella* infections, we determined the capacity of *Shigella* to survive and multiply in established *Bacteroides* cultures. The data show that inhibition occurred when *Shigella* was inoculated into *Bacteroides* cultures. If, for example, *Shigella* was inoculated into an exponential-phase *Bacteroides* 5 culture, its viable population, after 48 hr of incubation, was approximately 1,000-fold smaller than its population in a similarly inoculated DL medium control. This effect can be seen in Fig. 2. A

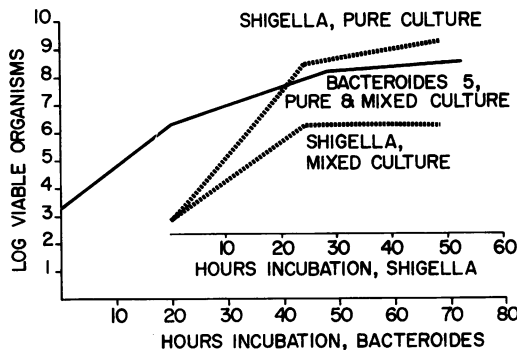

FIG. 2. *Growth curves for Shigella in DL medium in pure culture and in mixed culture when inoculated into an exponential-phase Bacteroides 5 culture.*

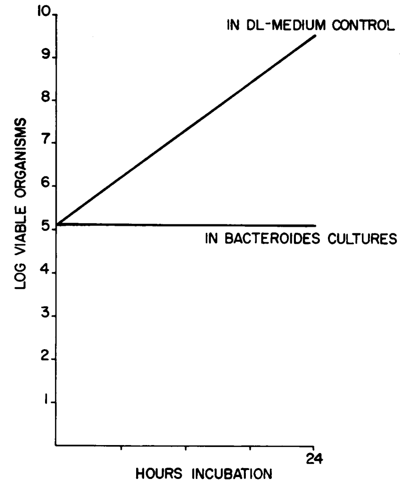

FIG. 3. *Differences in viable Shigella populations after 24 hr of incubation in DL medium and in stationary-phase Bacteroides cultures.*

comparison of the *Shigella* pure- and mixed-culture growth curves in Fig. 2 illustrates that the diminished *Shigella* population in mixture resulted from a decreased growth rate and a premature interruption of exponential growth.

Shigella failed to multiply at all in stationary-phase *Bacteroides* cultures. Figure 3 summarizes results obtained when *Shigella* was inoculated into 72-hr cultures of the five *Bacteroides* strains. The lines in the figure are not intended to represent growth curves but rather to illustrate differences in *Shigella* populations after 24 hr of incubation. The top line represents the mean viable *Shigella* population in DL medium controls, and the bottom line, the mean viable population in *Bacteroides* cultures. There was no increase in the *Shigella* population after incubation in the *Bacteroides* cultures. In control medium, however, *Shigella* attained a mean viable population of 3.4×10^9 organisms per ml. Similar results were obtained when the *Bacteroides* cultures were heat-killed (50 C for 30 min) prior to *Shigella* inoculation. Figure 4 summarizes results of these experiments. Although *Shigella* multiplied to some degree (approximately 10-fold) in the heat-killed cultures, when compared with controls, inhibition was clearly apparent.

To determine the mechanisms responsible for the inhibition of *Shigella*, stationary-phase *Bacteroides* cultures were analyzed for the presence of environmental factors previously shown to be involved with *Shigella* inhibition in liquid cultures (7, 9). The *p*H, oxidation-reduction potentials, and volatile fatty acid contents of 72-hr *Bacteroides* cultures were measured. Table 2 shows

that DL medium, before inoculation, had a *p*H of 6.82 and an E_h of −292 after 2 days of storage in the anaerobic chamber. As a result of *Bacteroides* growth, the *p*H of the culture medium decreased from 1.12 to 1.37 units but the E_h change was negligible. It is interesting that the E_h increased slightly. Acetic acid was produced in relatively high concentrations (from 0.209 to 0.267%) by all five *Bacteroides* strains, and propionic acid was produced in relatively low concentrations (from 0.015 to 0.058%) by four of the five strains.

The influence of each environmental change, alone and in combination with other changes, on *Shigella* growth was examined next. The results of these experiments are illustrated in Fig. 5, where the top line represents *Shigella* populations after 24 hr of incubation either in reduced DL medium or in DL medium under static conditions. The

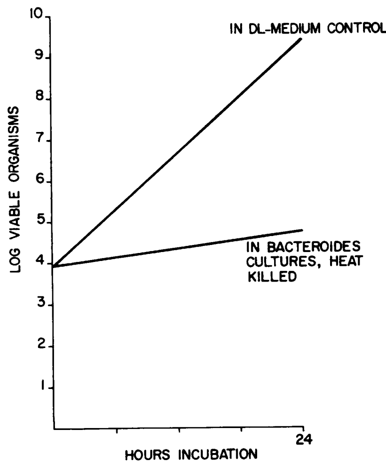

FIG. 4. *Differences in viable Shigella populations after 24 hr of incubation in DL medium and in heat-killed stationary-phase Bacteroides cultures.*

E_h of DL medium under static conditions, outside the anaerobic chamber, was +280 mv, whereas its E_h under reducing conditions was −292 mv. Since there was no difference in *Shigella* population sizes under either static or reducing conditions, both populations are represented by a single line. Lowered oxidation-reduction potential, in itself, had no inhibitory effect on *Shigella*. There was a marked decrease in the *Shigella* population when volatile acids, in average concentrations produced by the five *Bacteroides* strains, were added to reduced DL medium. Addition of volatile acids lowered the *p*H of the medium to 5.75. The viable *Shigella* population under these conditions was nearly 100,000 times smaller than its population in the DL medium control. Identical results were obtained when the *p*H of the medium was adjusted (with HCl) to 5.55, the mean *p*H recorded for 72-hr *Bacteroides* cultures. Both of these results are represented by the middle line in Fig. 5. The greatest degree of inhibition occurred, however, when volatile acids were added to the medium and, in addition, the *p*H of the medium was adjusted to 5.55, simulating conditions existing in *Bacteroides* cultures. In this case, illustrated by the bottom line in Fig. 5, the *Shigella* population was more than 200,000 times smaller than its population in the DL medium control. These results are similar to those obtained with *Shigella* in heat-killed, 72-hr *Bacteroides* cultures (see Fig. 4). In both cases, although there was approximately a 10-fold increase in the viable *Shigella* population after 24 hr of incubation, when compared with DL medium controls, multiplication was markedly inhibited.

Role of glucose in inhibition. Alteration of the glucose content of DL medium changed *Shigella* inhibition patterns in *Bacteroides* cultures. Figure 6 illustrates the *Shigella* population increases recorded after incubation in 72-hr *Bacteroides* 5 cultures with various glucose concentrations. The

TABLE 2. *Environmental changes resulting from Bacteroides growth in DL medium*

Culture	*p*H	O-R Potential[a]	Volatile fatty acids	
			Acetic acid	Propionic acid
		mv	%	%
None (initial values in medium)	6.82	−292	0	0
B1	5.70 (−1.12)[b]	−280 (+12)	0.267	0
B2	5.63 (−1.19)	−280 (+12)	0.228	0.043
B3	5.45 (−1.37)	−278 (+14)	0.209	0.058
B4	5.50 (−1.32)	−288 (+4)	0.240	0.015
B5	5.50 (−1.32)	−288 (+4)	0.266	0.055

[a] Oxidation-reduction potential expressed as E_h.
[b] Extent of changes is given parenthetically for the *p*H and O-R potential.

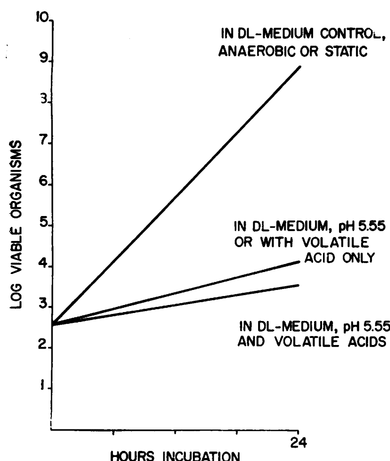

FIG. 5. *Influence of anaerobiasis, pH adjustment of DL medium, and volatile fatty acid addition to DL medium on the size of Shigella 24-hr populations.*

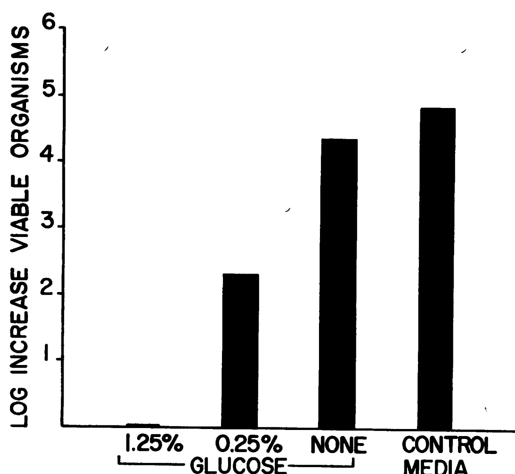

FIG. 6. *Influence of the initial glucose concentration of DL medium on the extent of Shigella growth in stationary-phase Bacteroides cultures.*

stippled bar on the right represents the mean viable *Shigella* population increase in the DL medium controls. Despite variations in the glucose concentration in the controls, *Shigella* populations increased to approximately the same extent. A single bar is used, therefore, to represent all three. The shaded bars in the figure represent *Shigella* population increases in the *Bacteroides* 5 cultures. An inverse relationship appeared to exist between the initial glucose concentration in the medium and the extent of *Shigella* multiplication in *Bacteroides* 5 cultures. In the culture initially containing 1.25% glucose, for example, little or no *Shigella* multiplication occurred. In cultures initially containing 0.25% glucose, the *Shigella* population increase was approximately 200-fold. In glucose-free cultures, the *Shigella* population increased 20,000-fold, nearly equaling its increase in control media.

In an effort to explain differences in inhibitory effects, the volatile fatty acid content and *p*H values of *Bacteroides* 5 cultures with different glucose concentrations were measured. Results showed that all cultures contained approximately equal quantities of volatile fatty acids (from 0.04 to 0.05 N), indicating that the *Bacteroides* strain produced the acid both in the presence and in the absence of glucose. The *p*H of the cultures varied considerably, however, depending on the initial glucose concentration. In *Bacteroides* 5 cultures with a glucose concentration of 1.25%, the *p*H was 5.5; in cultures with a 0.25% concentration, the *p*H was 5.9; and in glucose-free cultures, the *p*H was 7.3. The *p*H of the *Bacteroides* 5 cultures varied, therefore, depending on initial glucose concentration in the culture medium. Volatile fatty acids accumulated to approximately the same extent despite differences in the glucose concentration. The greater degree of *Shigella* inhibition in *Bacteroides* cultures initially containing glucose was attributed to the toxicity of the volatile acids at low *p*H (see Fig. 5). At the higher *p*H of the glucose-free culture, the acids had little or no toxic effect on *Shigella*.

DISCUSSION

The extent to which *Shigella* growth is inhibited in a *Bacteroides* culture initially containing glucose depends upon the age of the culture. No inhibition occurs when *Shigella* and *Bacteroides* are simultaneously inoculated into liquid culture medium. Inhibition occurs only after *Bacteroides* has undergone some degree of multiplication prior to *Shigella* inoculation. Inhibition is less pronounced after inoculation of *Shigella* into an exponential-phase *Bacteroides* culture than into a stationary-phase culture. During growth, *Bacteroides* strains produce acids, presumably as metabolic end products, that interfere with *Shigella* multiplication. If *Shigella* is inoculated into *Bacteroides* cultures before the acids are produced, multiplication occurs without interference, and *Shigella* attains a maximal population equivalent to that observed in control medium (see Fig. 1). If, on the other hand, acids have accumulated in the *Bacteroides* cultures prior to *Shigella* inoculation, then inhibition is apparent. The extent of the inhibition appears to be dependent upon the con-

centration of acids present in the medium. At a low concentration a decrease in the *Shigella* multiplication rate is observed, whereas at higher concentrations *Shigella* fails to multiply at all.

Inhibition appears to be primarily, but not entirely, an H ion effect. Adjustment of the liquid medium (with HCl) to *p*H 5.55, the mean *p*H recorded in stationary-phase *Bacteroides* cultures, results in a marked inhibition of *Shigella* growth (see Fig. 5). A greater degree of inhibition is observed when, in addition to *p*H adjustment, volatile acids are added to the medium. Under these conditions, the inhibition is equivalent to that observed in heat-killed *Bacteroides* cultures. At *p*H 5.55, *Shigella* inhibition is more pronounced in the presence than in the absence of volatile acids, which indicates that volatile acids, per se, contribute to the inhibitory effect.

It becomes apparent, in comparing Fig. 3 and 4, that *Shigella* inhibition is greater in living than in heat-killed *Bacteroides* cultures. The greater inhibition in living cultures may be a consequence of continued acid production. This is evidenced by the observation that the *p*H of *Bacteroides* cultures decreases at an almost constant rate during growth. A 96-hr *Bacteroides* culture, at a lower *p*H, for example, is more inhibitory for *Shigella* than a 72-hr culture.

It is interesting that *Bacteroides* strains produce acetic and propionic acids as metabolic end products in either the presence or the absence of glucose. In the absence of a fermentable carbohydrate, the organisms presumably utilize peptones as carbon energy sources. The formation of nitrogenous residues as a result of peptone utilization offsets the decrease in *p*H usually associated with the accumulation of acid end products in bacterial cultures. Consequently, the *p*H of *Bacteroides* cultures does not decrease as a result of cell metabolism. At the relatively high *p*H of glucose-free cultures, *Shigella* multiplies almost as well as in control medium, although volatile acids are present (see Fig. 6). These results reaffirm the importance of the relationship between volatile acids and *p*H in the inhibition of *Shigella* growth. At *p*H levels above neutrality, the volatile acids are almost entirely in the dissociated state. At lower *p*H levels, the proportion of undissociated acid increases. Previous work demonstrated that undissociated volatile acid molecules are responsible for *Shigella* inhibition (8). In medium of high *p*H, volatile acids are, therefore, ineffective in inhibiting *Shigella* multiplication. Only in medium of a low *p*H, containing a large proportion of undissociated acid molecules, is *Shigella* growth inhibited.

Our primary objective in studying interactions between *Shigella* and intestinal bacteria is an understanding of the protection afforded by the indigenous intestinal flora in preventing *Shigella* infections. From the information obtained in this study, what can be inferred about the relationships existing between *Shigella* and *Bacteroides* in vivo? It is well known that volatile acids are normally present in the intestines of animals and that the normal flora is largely responsible for their production (2, 3, 11). Our data show that *Bacterioides* produces volatile acids in vitro in the presence or absence of a fermentable carbohydrate. It is reasonable to assume, therefore, that these bacteria, which are the predominant organisms in the intestines of man and many animals, are the principal acid producers in the intestines. In the presence of volatile acids and at the *p*H levels existing in the intestine under normal circumstances, *Shigella* may not be able to survive and become established. This hypothesis is currently being tested in germ-free animals and will be the subject of another report.

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