INFECTION AND IMMUNITY, Aug. 1972, p. 168-173

Vol. 6, No. 2
Printed in U.S.A.

Experimental *Shigella* Infections in Laboratory Animals

I. Antagonism by Human Normal Flora Components in Gnotobiotic Mice[1, 2]

BRUCE R. MAIER AND DAVID J. HENTGES

Department of Microbiology, University of Missouri School of Medicine, Columbia, Missouri 65201

Received for publication 21 March 1972

Germfree mice were associated with selected species of human intestinal bacteria and then challenged with a streptomycin-resistant *Shigella flexneri* strain. Antagonism against *Shigella* was most pronounced in mice associated with *Escherichia coli* and least pronounced in mice associated with *Bacteroides fragilis*. A moderate degree of antagonism could be demonstrated in mice associated with either *Streptococcus faecalis* or *Bifidobacterium adolescentis*. *Shigella* persisted in the cecal contents of *E. coli*-associated mice at very low, stable levels. *Shigella* populations were reduced to levels below detection in the ceca of mice diassociated with *E. coli* and *Bacteroides*. Upon subsequent administration of streptomycin, *Bacteroides* disappeared from the ceca. The *E. coli* population was greatly reduced, and *Shigella* reappeared at very high population levels as an apparent recombinant which resembled *E. coli* biochemically. A streptomycin-resistant *E. coli* population subsequently emerged and became dominant in the ceca. *Shigella* concomitantly declined to levels below detection.

Several studies have dealt with the significance of the indigenous intestinal flora in natural protection against *Shigella* infections. Freter (9) found that the oral administration of antibiotics eliminated the intestinal flora and rendered mice and guinea pigs susceptible to infection with both *Shigella flexneri* and *Vibrio cholerae*. Introduction of *Escherichia coli* into the intestines of these animals resulted in the elimination of the pathogens. Formal et al. (8) used germfree guinea pigs to demonstrate that *E. coli* prevented the establishment of *S. flexneri* in the intestines of the animals. Hentges and Freter (14) found that several different kinds of intestinal bacteria, including *E. coli*, *Aerobacter aerogenes*, and *Proteus* sp. interfered with the multiplication of *S. flexneri* in the intestines of antibiotic-treated mice.

In our laboratory, we found that *S. flexneri* is also inhibited in vitro by various components of the human indigenous intestinal flora. Fourteen of fifteen strains of coliform species inhibited *Shigella* multiplication in mixed culture (12, 13). Inhibition was due to the production of formic and acetic acids by the coliform strains, which, under the reducing conditions and *p*H levels present in the cultures, were bactericidal for *Shigella*. Because of the predominance of anaerobic bacteria in the intestines of man and many animals (5, 7, 21), the inhibitory activity of *Bacteroides fragilis* strains against *Shigella* was also investigated (15). Antagonism against *Shigella* was observed when the pathogen was inoculated into established *Bacteroides* cultures multiplying in media containing glucose. Volatile fatty acids produced by *Bacteriodes* and the low *p*H that developed in the culture media interfered with the multiplication of *Shigella*. *Bacteriodes* similarly produced volatile fatty acids in glucose-free media, but the *p*H of the media remained above 7.0 during growth. At this *p*H level, the acids had little or no toxic effect on *Shigella* and inhibition was not observed.

Since both facultative and strictly anaerobic bacteria were shown to be capable of inhibiting *Shigella* multiplication under certain conditions in vitro, we were interested in determining if organisms from the two major categories of bacteria were capable of antagonizing *Shigella* multiplication in vivo. We have studied the interactions between *S. flexneri* and selected components of the human intestinal flora in the ceca

[1] Presented in part at the Annual Meeting of the American Society for Microbiology, Minneapolis, 2-7 May, 1971.

[2] Taken from a dissertation presented to the Graduate School, University of Missouri, by B. R. Maier in partial fulfillment of requirements for the Ph.D. degree.

of germfree mice. In this paper, we report results of experiments which demonstrate that various degrees of antagonism are exerted against *Shigella* by indigenous flora components in vivo.

MATERIALS AND METHODS

Microorganisms. All strains of bacteria used in these studies were isolated from human beings. The *S. flexneri* species, a streptomycin-resistant strain, was used in previous studies (12–15) as was *E. coli* (13, 14) and *B. fragilis* (15). *Bifidobacterium adolescentis* was isolated from the feces of a 12-week-old, breast-fed infant, and was identified according to criteria outlined by the Anaerobe Laboratory at Virginia Polytechnic Institute and State University (19). *Streptococcus faecalis*, isolated from the feces of an adult, was identified by tests outlined by Cowan and Steel (3).

Anaerobic methods. Strict anaerobes, *Bacteroides* and *Bifidobacterium*, were cultured in a vinyl anaerobic glove box isolator described by Aranki et al. (1). The chamber contained a gas mixture consisting of nitrogen, 85%; hydrogen, 10%; and carbon dioxide, 5%, which was circulated over a palladium catalyst to remove trace oxygen. A constant temperature of 37 C was maintained in the chamber with an electrical heating element and a thermoregulator.

Media. Veal infusion agar (BBL) containing 1 mg of streptomycin/ml was used for plating *Shigella* cultures. An enriched Trypticase soy agar (ETSA), prepared as described by Aranki et al. (1), was used for plating *Bacteroides* cultures. ETSA plates were prepared with an agar overlay containing palladium black to lower the surface oxygen tension.

A new medium was devised for the cultivation of *Bifidobacterium*. Bifidobacterium-Lactobacillus agar (BLA) contains 4.5% Trypticase soy agar, 0.05% galactose, 0.05% cellobiose, 0.4% lactose, 0.5% yeast extract, 0.1% placenta powder, and 0.3% NaCl. The *p*H of the medium was adjusted to 5.7 with 1 N HCl. Plates were prepared with an agar overlay containing palladium black. This medium proved to be both differential and selective for *Bifidobacterium* under anaerobic conditions. Large *Bifidobacterium* colonies could easily be distinguished from the microcolonies of other species within 48 hr of incubation.

Streptococcus was cultured on M-Enterococcus agar (BBL) and *E. coli* on veal infusion agar and MacConkey's agar. Data from preliminary studies showed that there was no statistically significant difference in *E. coli* colony counts on veal infusion-agar and MacConkey's agar.

Germfree animals and techniques. Germfree mice, strain ARS HA/ICR, were purchased from Sprague-Dawley (Madison, Wis.). The animals were kept in Trexler-type isolators and were housed in plastic cages on shredded corncob bedding. Mice were maintained on vitamin-enriched diet L-356 (General Biochemicals Co.) which is formulated for germfree animals. Food and water were provided ad libitum except for withdrawal before association of the mice with bacteria.

The germfree state of newly arrived mice was tested before each experiment. Tests for contamination were performed by collecting a fresh fecal pellet and swabbing the skin of two mice in each cage. These specimens were streaked onto the surface of prereduced blood-agar slants which were covered with 4 ml of reduced liquid ETSA medium. These tubes, which supported the growth of strict anaerobes, were incubated in the anaerobic isolator. A duplicate set of slants, which were not prereduced, were similarly streaked and incubated aerobically. All tubes were incubated for 48 hr.

Association of mice with bacteria. Food and water were withdrawn 10 hr before association of the mice with bacteria. Fresh plate cultures of the organisms were washed and diluted with water to give approximately 10^7 viable organisms/ml. Results from preliminary studies showed that all organisms used in the studies survived well in the sterile water for at least 3 hr. The bacterial suspensions were added to water bottles inside the germfree isolators. Animals were allowed to drink the bacterial suspensions for a period of 2 hr, after which normal food and water were returned to the cages.

Assay of cecal contents. Animals were removed from the gnotobiotic isolators in sterile cages. They were sacrificed by vertebral dislocation, and their ceca were immediately excised. Portions of the cecal contents were aseptically added to tubes of sterile saline for weighing. Tenfold serial dilutions were then prepared from the contents in saline. Samples (1 ml) from the various saline dilutions were flooded on to predried agar plates (11) which were incubated for 3 days before colonies were counted. Assays of *Bacteroides* and *Bifidobacterium* were performed entirely within the anaerobic isolator. Colony counts were performed with an electronic colony counter (New Brunswick Scientific Co., N.J.). Portions of the cecal contents were weighed and then dried for 48 hr at 90 C, after which they were weighed again to determine moisture content. All plate counts were expressed as viable organisms per gram (dry weight) of cecal content. Typical colonies of the various bacteria studied were picked from the plates and identified by standard procedures.

Delivery of oral streptomycin. Five milligrams of streptomycin sulfate per milliliter was added to the sterile drinking water supplied to the mice. The animals readily drank the water.

RESULTS

Monoassociated mice. The germfree mice were monoassociated with each bacterial species to determine if the test strains could be established in these animals. Plate counts were prepared from the cecal contents of the animals at various times after association. Figure 1 illustrates the multiplication of *Shigella* in the ceca of monoassociated mice. *Shigella* became established within 6 hr after oral inoculation, and attained maximal levels of approximately 10^{10} viable cells/g (dry weight) of the cecal material within 24 hr. Similar population levels of 10^{10} viable cells/g

(dry weight) were found in the ceca of mice monoassociated with *E. coli*, *Bifidobacterium*, and *Streptococcus*. Of the species examined, *Bacteroides* attained the highest population levels in monoassociated mice. Figure 1 shows that after 4 days, *Bacteroides* attained a maximal population level of 10^{11} organisms/g of cecal material.

Shigella challenge to associated mice. To determine which of the intestinal flora components inhibit *Shigella* multiplication in vivo, mice were monoassociated with the bacteria and then challenged with *Shigella* 8 days later. Differential counts of *Shigella* and the test strain were prepared from the cecal material at varying time intervals after challenge. In no case did the presence of *Shigella* in the ceca influence the population levels of the test strains. Figure 2 illustrates the counts obtained when mice, as-

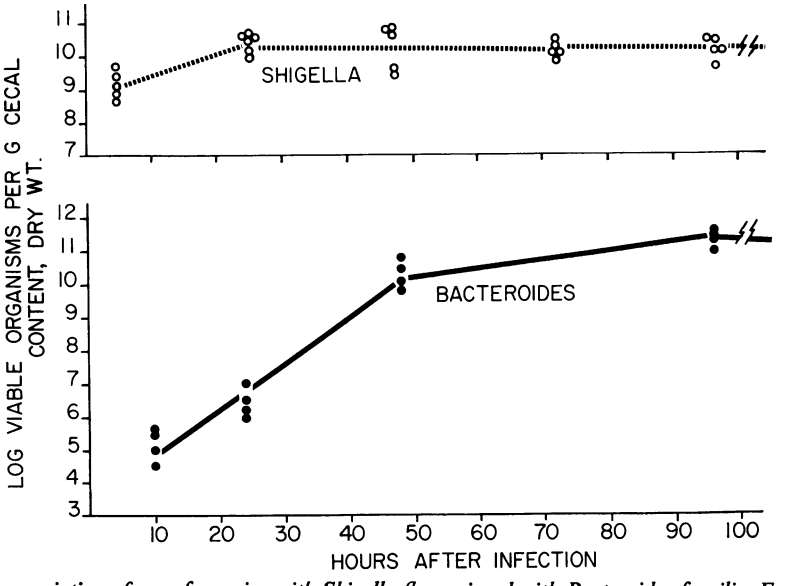

FIG. 1. *Monoassociation of germfree mice with Shigella flexneri and with Bacteroides fragilis. Each dot represents results obtained from one experimental animal. Population levels similar to those of Shigella were observed in mice monoassociated with Bifidobacterium adolescentis, Escherichia coli, and Streptococcus faecalis.*

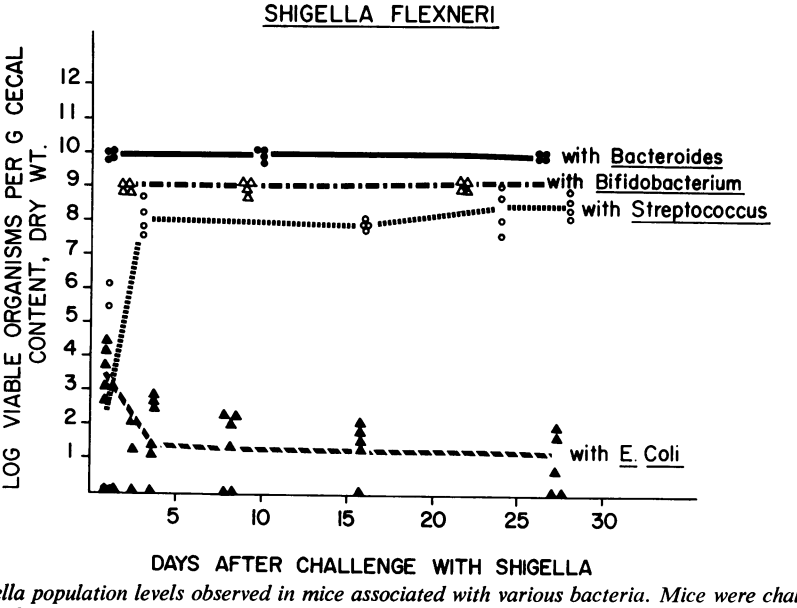

FIG. 2. *Shigella population levels observed in mice associated with various bacteria. Mice were challenged with Shigella 8 days after monoassociation. Each dot represents results obtained from one experimental animal.*

sociated with the test strains, were challenged with *Shigella*. *Shigella* multiplied in *Bifidobacterium*-associated mice to attain population levels of 10^9 viable organisms/g of cecal material. This represents approximately a 10-fold decrease when compared with population levels attained in mice monoassociated with *Shigella*. Figure 2 shows that *Shigella* multiplied in the ceca of animals associated with *Streptococcus* to attain population levels of approximately 10^8 organisms/g, which represents a 10-fold to 100-fold decrease when compared with *Shigella* populations in monoassociated mice. Cecal counts obtained from mice monoassociated with *Bacteroides* and subsequently challenged with *Shigella* are also illustrated in Fig. 2. *Shigella* was not inhibited in the ceca of *Bacteroides*-associated mice, but attained population levels of 10^{10} viable cells/g. This population density is equivalent to *Shigella* levels in monoassociated mice. *Shigella* failed to multiply in mice associated with *E. coli*. The *Shigella* population declined to approximately 10^1 to 10^2 viable organisms/g of cecal material (Fig. 2). *Shigella* persisted at this low population level for 58 days, the duration of the experiment.

To summarize, in vivo antagonism against *Shigella* was most pronounced in mice associated with *E. coli*. A moderate degree of antagonism against *Shigella* could be demonstrated in mice associated with either *Streptococcus* or *Bifidobacterium*, but no antagonism could be demonstrated in mice associated with *Bacteroides*.

Disassociation, Shigella challenge, and streptomycin delivery. Because *Shigella* was inhibited to the greatest degree by *E. coli*, and since *Bacteroides* is a dominant organism in the intestine, a simplified gut ecosystem was created by disassociating germfree mice with these normal flora components and then challenging the mice with *Shigella*. *Shigella* populations fluctuated erratically for a period of 2 weeks after challenge and then began to decline (Fig. 3). After 24 days, *Shigella* could no longer be recovered from the cecal contents of the animals. We were interested at this point in determining if the administration of streptomycin to these mice would permit the streptomycin-resistant *Shigella* to multiply by eliminating the sensitive *E. coli* and *Bacteroides* strains. Streptomycin was therefore administered continuously in the drinking water of four mice kept in a separate isolator. *Bacteroides* immediately disappeared from the cecal contents when streptomycin was administered (Fig. 3). The *E. coli* population decreased to approximately 10^6 organisms/g, a 10,000-fold reduction from pre-streptomycin levels. Concomitantly, streptomycin-resistant *Shigella* reappeared and multiplied to attain a population level of 10^{10} organisms/g. A streptomycin-resistant population of *E. coli* then emerged and became dominant in the ceca. The rise of this population was coincident with the decline of *Shigella* populations to levels below detection. Almost identical results were obtained when the experiment was repeated. *Shigella* could not be isolated from the ceca of mice which did not receive streptomycin (Fig. 3). In these untreated animals, both the *E. coli* and *Bacteroides* populations remained at high levels.

After streptomycin administration, *Shigella* colonies became dense and opaque so that they

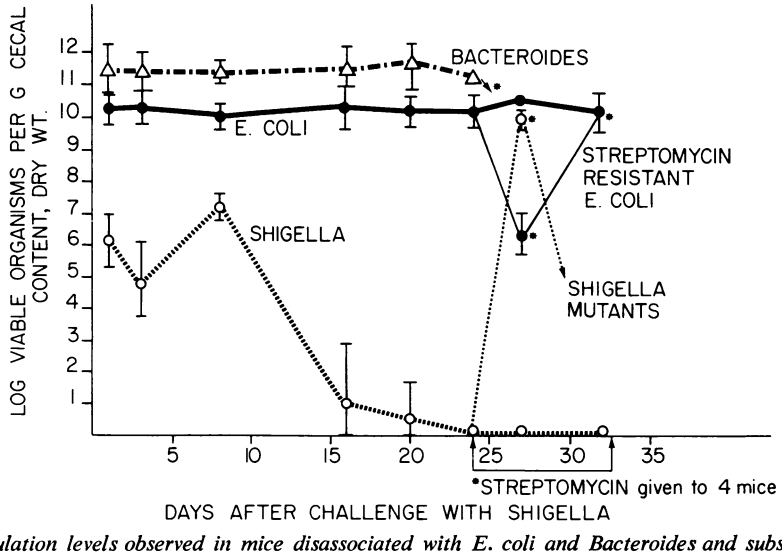

FIG. 3. *Population levels observed in mice disassociated with E. coli and Bacteroides and subsequently challenged with Shigella. Asterisks indicate mean results obtained from four mice given streptomycin. Other symbols represent mean results from eight mice. Vertical lines indicate ranges of observations.*

closely resembled *E. coli* colonies. The usual characteristics of *Shigella* colonies (translucent, smooth, green-gold) were not observed. Representative aberrant colonies were picked and subjected to the tests listed in Table 1. The table summarizes results of tests performed on *Shigella* and *E. coli* colonies recovered before and after streptomycin administration. The *Shigella* strain recovered after streptomycin administration demonstrated an increased saccharolytic activity as compared to the pre-streptomycin strain. This aberrant strain resembled *E. coli* in the fermentation of lactose, sucrose, and mannitol and the production of indole from tryptophan. Although the *Shigella* strain, recovered after antibiotic administration, was not streptomycin-dependent, it grew very slowly in the absence of streptomycin. In spite of the aberrant biochemical reactions, the *Shigella* strain retained its serological identity as *S. flexneri*.

DISCUSSION

The results of this study show that *S. flexneri* and four selected components of the human enteric flora can be independently established in the intestines of germfree mice. All of these bacteria attained population levels that ranged from between 10^{10} and 10^{12} viable organisms/g of cecal material. Thus, the germfree mouse provides an in vivo test system which allows the ecology of *Shigella* interactions with other intestinal bacteria to be studied without pretreatment of the host.

Contrary to our expectations and the speculations of other workers (18), the anaerobic bacteria *Bacteroides* and *Bifidobacterium*, which are numerically dominant in the intestine, did not, by themselves, inhibit *Shigella* to any extent in vivo. It is interesting that, of the normal flora components studied, only *E. coli* was able to antagonize *Shigella* to a significant degree. These observations are consistent with the reports of others in which *E. coli* was shown to interfere with the multiplication of *Shigella* both in vitro and in vivo (13, 14).

The persistence of *Shigella*, in association with *E. coli*, in the ceca of the animals for long periods of time suggests that *Shigella* are sequestered in some way from the antagonistic effects of *E. coli* and are periodically shed into the lumen of the intestine. We are, therefore, investigating the localization of *Shigella* in the intestinal tissues of *E. coli*-associated mice and the persistence of *Shigella* in the feces of these animals. This condition in mice may resemble the *Shigella* carrier state in humans and may provide a model for the study of asymptomatic shigellosis. The high incidence of *Shigella* infections in human beings without the symptoms of dysentery has been reported by several investigators (10, 16). Because of the importance of the asymptomatic carrier as a reservoir of infection, an animal model of the carrier state may provide a valuable tool for the study of latent *Shigella* infections.

Acquisition by *Shigella* of certain biochemical characteristics associated with *E. coli*, after the administration of streptomycin, suggests that a *Shigella–E. coli* recombination may have occurred within the mouse gut. Luria (17) reported the in vitro conjugation of *Shigella* and *E. coli* to produce recombinants.

A number of investigators (4, 6, 20) have

TABLE 1. *Characterization of Shigella flexneri and Escherichia coli strains*

Characteristic	Strain			
	E. coli (parent)	*E. coli* (streptomycin-resistant)	*Shigella* (parent)	*Shigella* (hybrid)
Glucose	Acid, gas	Acid, gas	Acid	Acid, gas
Lactose	Acid, gas	Acid, gas	–	Acid, gas (delayed)
Sucrose	Acid, gas	Acid, gas	–	Acid
Mannitol	Acid, gas	Acid, gas	Acid	Acid, gas (weak)
Salicin	Acid, gas	Acid, gas	–	–
Indol	+	+	–	+
Lysine decarboxylase	+	+	–	–
Voges-Proskauer	–	–	–	–
Urease	–	–	–	–
Motility	+	+	–	–
Streptomycin resistance	–	+	+	+ (grows slowly in absence)
Agglutination in *Shigella* anti-B (*flexneri*)	–	–	+	+

reported strains of bacteria, isolated from clinical specimens, which possess the experimental pathogenicity of *Shigella* but resemble *E. coli* biochemically. These organisms have been called "parashigellas" and may represent a new class of pathogens. This adds a potential dimension to the difficult task of tracing and identifying enteric pathogens. For example, the *Shigella–E. coli* hybrid that we isolated would probably not be classified as a pathogen if routine diagnositc procedures were utilized. Instead, because of its biochemical characteristics, it would be identified as a slow lactose-fermenting *E. coli*. Hybridization occurring in vivo may play a significant role, therefore, in the evolution of the *Enterobacteriaceae* and the origin of intermediate strains of pathogenic bacteria.

ACKNOWLEDGMENTS

This work was done under the sponsorship of the Commission on Enteric Infection of the Armed Forces Epidemiological Board, and was supported by the U.S. Army Medical Research and Development Command, under research contract no. DA 17-68-C-8162.

LITERATURE CITED

1. Aranki, A., S. A. Syed, E. B. Kenney, and R. Freter. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. Appl. Microibol. 17:568–578.
2. Bohnhoff, M., C. P. Miller, and W. R. Martin. 1964. Resistance of the mouse's intestinal tract to experimental *Salmonella* infection. I. Factors which interfere with the initiation of infection by oral inoculation. J. Exp. Med. 120:805–816.
3. Cowan, S. T., and K. J. Steel. 1966. Manual for the identification of medical bacteria. Cambridge University Press, London.
4. Cox, C. D., and G. I. Wallace. 1948. A study of *Shigella* isolated in India and Burma, with special reference to two previously undescribed serotypes. J. Immunol. 60:465–473.
5. Drasar, B. S., M. Shiner, and G. M. McLeod. 1969. Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. Gastroenterology 56:71–79.
6. Ewing, W. H., H. W. Tatum, and B. R. Davis. 1957. The occurrence of *E. coli* serotypes associated with diarrheal disease in the United States. Pub. Health Rep. 15:118–135.
7. Finegold, S. M., V. L. Sutter, J. D. Boyle, and K. Shimada. 1970. The normal flora of ileostomy and transverse colostomy effluents. J. Infect. Dis. 122:376–381.
8. Formal, S. B., G. Dammin, H. Sprinz, D. Kundel, H. Schneider, R. E. Horowitz, and M. Forbes. 1961. Experimental *Shigella* infections. V. Studies in germ-free guinea pigs. J. Bacteriol. 82:284–287.
9. Freter, R. 1956. Experimental enteric *Shigella* and *Vibrio* infections in mice and guinea pigs. J. Exp. Med. 104:411–418.
10. Hardy, A. V., and J. Watt. 1944. The acute diarrheal diseases. J. Amer. Med. Ass. 124:1173–1179.
11. Hentges, D. J. 1962. A simplified plating technique for bacteriological examination of specimens of urine. Amer. J. Clin. Pathol. 38:304–305.
12. Hentges, D. J. 1967. Inhibition of *Shigella flexneri* by the normal intestinal flora. I. Mechanisms of inhibition by *Klebsiella*. J. Bacteriol. 93:1369–1373.
13. Hentges, D. J. 1969. Inhibition of *Shigella flexneri* by the normal intestinal flora. II. Mechanisms of inhibition by coliform organisms. J. Bacteriol. 97:513–517.
14. Hentges, D. J., and R. Freter. 1962. *In vivo* and *in vitro* antagonism of intestinal bacteria against *Shigella flexneri*. I. Correlation between various tests. J. Infect. Dis. 110: 30–37.
15. Hentges, D. J., and B. R. Maier. 1970. Inhibition of *Shigella flexneri* by the normal intestinal flora. III. Interactions with *Bacteroides fragilis* strains in vitro. Infect. Immunity 2:364–370.
16. Higgins, A. R., T. M. Floyd, and M. A. Kader. 1955. Studies in shigellosis. III. A controlled evaluation of a monovalent *Shigella* vaccine in a highly endemic environment. Amer. J. Trop. Med. Hyg. 4:271–280.
17. Luria, S. E., and J. W. Burrous. 1957. Hybridization between *Escherichia coli* and *Shigella*. J. Bacteriol. 74:461–476.
18. Miller, C. P., and M. Bohnhoff. 1963. Changes in the mouse's enteric microflora associated with enhanced susceptibility to *Salmonella* infection following streptomycin treatment. J. Infect. Dis. 118:59–66.
19. Outline of clinical methods in anaerobic bacteriology. 1970. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.
20. Szturm-Rubinsten, S., D. Piechaud, H. D'Hauteville, and H. Sarrat. 1970. Variete gazogene du serotype provisoire de *Shigella*: 2000–53. Ann. Inst. Pasteur 119:320–322.
21. Zubrzycki, L., and E. H. Spaulding. 1962. Studies on the stability of the normal human fecal flora. J. Bacteriol. 83:968–974.