Effect of the Normal Microbial Flora on the Resistance of the Small Intestine to Infection

GERALD D. ABRAMS AND JANE E. BISHOP

Department of Pathology, The University of Michigan Medical School, Ann Arbor, Michigan

Received for publication 17 August 1966

ABSTRACT

ABRAMS, GERALD D. (The University of Michigan Medical School, Ann Arbor), AND JANE E. BISHOP. Effect of the normal microbial flora on the resistance of the small intestine to infection. *J. Bacteriol.* 92:1604-1608. 1966.—Mucosal structure in the small intestine is known to be influenced by the normal microbial flora. This suggests that mucosal resistance to invasion by enteric pathogens might also be affected by the flora. To assess this possibility, germ-free and conventional mice were challenged with *Salmonella typhimurium*, and both the growth of organisms within the intestinal lumen and the translocation to mesenteric lymph nodes were studied quantitatively. There were significantly more organisms 24 hr after intragastric challenge in the mesenteric nodes of germ-free animals than in those of conventional ones. However, since intraluminal growth in the intestine was also greater in germ-free animals, no conclusion could be drawn about mucosal resistance per se. Results were similar when the challenge was intraduodenal. However, when intestinal emptying was prevented by ileal ligation before challenge, both intraluminal growth and translocation of *S. typhimurium* were equal in the two groups of mice. It is concluded from these data, as well as from preliminary dye studies of intestinal motility, that the normal flora does not influence mucosal resistance directly, but may alter enteric infection by affecting intestinal emptying.

Defense of the host against infection is a well-recognized function of the normal intestinal flora. This protective property is usually explained on the basis of inhibitory effects exerted by the resident flora upon a potential invader, by virtue of their competing for limited nutrients (8) or through their producing of antibacterial substances (3, 13). However, the normal flora also has a more direct impact upon the host, being responsible, to a striking degree, for the development of a number of anatomical and physiological traits of the host ordinarily considered "normal." This is particularly true within the small intestine, where it has been demonstrated, by comparison of conventional with germ-free animals, that the lamina propria acquires its usual structure as a result of exposure to the normal flora, that the life cycle of mucosal cells is influenced strikingly by the flora, and that even the surface area is affected by the presence or absence of the flora (1, 11). In view of these facts, it appeared that the flora might also have a direct influence on mucosal resistance to infection, and

thus play a role distinct from its antibacterial function within the intestinal lumen. The purpose of this study was to assess this possibility by comparing infection with *Salmonella typhimurium* in germ-free and conventional mice, with the extent of translocation of organisms from the intestinal lumen to the mesenteric lymph nodes serving as an index of the resistance of the mucosa in the two groups of animals.

MATERIALS AND METHODS

Cultures. *S. typhimurium* strain ETS 9 was employed. The challenge organisms were grown in Brain Heart Infusion (BHI) broth for approximately 22 hr at 37 C. A count of viable organisms was performed on each culture.

Animals. CD-1 Swiss mice (Charles River Breeding Laboratories) weighing 25 to 35 g were used. The germ-free animals were reared in isolators by use of standard gnotobiotic methods (2), whereas their conventional counterparts were raised in the open laboratory by use of an identical cage arrangement. Both groups of animals were fed an autoclaved L-356 diet (General Biochemicals Corp., Chagrin Falls,

Ohio) *ad lib*. In any given experiment, the germ-free and conventional animals were of matched ages and included approximately equal numbers of males and females.

Method of challenge. Before challenge, all animals were fasted overnight and had free access to water. The appropriate number of organisms, suspended in 0.5 ml of BHI, was administered to each animal by means of a fine polyethylene catheter attached to a 27-gauge needle. Intragastric challenge was accomplished simply by peroral intubation of unanesthetized mice. For intraduodenal challenge, each mouse was anesthetized with ether and was also intubated perorally. Then, via a small epigastric incision, intubation of the duodenum was accomplished under direct vision by manipulating the catheter through the stomach, past the pylorus, and into the first part of the duodenum. Immediately after challenge, the catheter was withdrawn, the incision was closed with interrupted sutures, and the animal was allowed to regain consciousness quickly. In the appropriate experiments, ileal ligation just proximal to the cecum was accomplished via the same incision prior to challenge. All animals were allowed free access to food and water after challenge. For the experiments lasting 24 hr after challenge, the germ-free animals were challenged within their isolators and removed just before sacrifice. Cultures for these latter experiments were sealed in glass ampoules and taken into the isolators via a peracetic acid lock. For the experiments involving duodenal intubation and survival for only 16 hr or less, both germ-free and conventional animals were challenged in the open laboratory by use of killed aseptic surgical technique, and were maintained thereafter in sterile cages for the brief experimental period.

Bacterial counts. At the end of the experimental period, animals were killed by aseptic cardiac puncture and exsanguination under ether anesthesia. Appropriate 10-fold serial dilutions of heparinized heart blood were prepared for quantitative plating. The entire mesenteric lymph node complex was removed aseptically from each animal and ground in saline in a tissue homogenizer; the homogenate was plated for bacterial enumeration. The small bowel was removed, minced in 10 ml of saline with its entire content, and placed in a stoppered flask. After 0.5 hr of agitation on a reciprocal shaker, dilutions of the supernatant fluid were prepared. All materials for quantitative culture were spread on the surface of MacConkey Agar, in duplicate plates. Enumeration of colonies was performed after 24 hr of incubation at 37 C.

RESULTS

The first series of experiments was designed to compare the number of salmonellae penetrating the mucosa in germ-free and conventional mice within the first 90 min after challenge. This short interval was selected because of reports elsewhere that certain other microorganisms appear in mesenteric lymph and in blood within moments of their introduction into the gastrointestinal

tract (9, 12). Furthermore, the brevity of the test period insured that the mucosa of all animals in both groups would be exposed to the same number of challenge organisms, there being insufficient time for significant multiplication within the lumen of the bowel. In an initial experiment, five germ-free and five conventional mice were challenged intraduodenally with 10^9 organisms. At the termination of the test interval, 9 of the 10 animals (the single exception being a conventional mouse which may have been traumatized during the intubation) were found to have completely negative cultures of blood and mesenteric lymph nodes. In subsequent experiments, the test interval was extended to 4 hr, with only sporadic cultural evidence of translocation of *Salmonella* from intestinal lumen to mesenteric lymph nodes.

Because these short intervals obviously did not allow meaningful comparison of the two groups, in the next series of experiments the animals were allowed to survive 24 hr after challenge with 10^9 organisms. Since the germ-free animals were to be maintained within their isolators for the test period, the intragastric route was chosen for challenge of all animals in the series as a matter of convenience. The longer test interval proved to be sufficient for the appearance of detectable numbers of *S. typhimurium* in the lymph nodes of mice in both groups. As seen in Table 1, the translocation of organisms during this time was almost 50-fold greater in the germ-free than in the conventional mice; the difference between the two means is highly significant statistically. Furthermore, the results of the blood cultures also attested to the greater spread of infection in the germ-free mice. *S. typhimurium* was recovered from the blood of all but one germ-free animal, the mean count being 1.9×10^3 per milliliter of blood, whereas all blood cultures were negative in the conventional mice. However, despite these striking differences in the extent of spread of the infection beyond the intestine in the two groups, no conclusions could be drawn as to a difference in mucosal resistance per se. As shown in Table 1, the environment provided by the intestine of the germ-free animals allowed for the multiplication and maintenance, during the test interval, of far greater numbers of *S. typhimurium* than in conventional animals. This difference alone, rather than any deficiency in germ-free mice of the mucosal barrier to invasion, could have accounted for a greater spread of infection.

In the next series of experiments, therefore, an attempt was made to create a situation in which the number of *S. typhimurium* in the intestine during the experiment would be similar in the

TABLE 1. Geometric mean counts of *Salmonella typhimurium* ETS 9 24 hr after intragastric challenge with 10^9 organisms

Status and no. of mice	Organisms in small intestine	Organisms in mesenteric node
Germ-free (5)	8.6×10^9	8.9×10^3
Conventional (6)	3.0×10^3 ($P < .01$)	1.9×10^2 ($P < .02$)

TABLE 2. Geometric mean counts of *Salmonella typhimurium* ETS 9 16 hr after intraduodenal challenge with 10^7 organisms

Status and no. of mice	Organisms in small intestine	Organisms in mesenteric node
Germ-free (8)	6.1×10^8	8.1×10^2
Conventional (5)	4.1×10^4 ($P < .02$)	2.0×10^1 ($P < .01$)

germ-free and conventional mice. The possible effect of a difference in gastric emptying time in the two groups was eliminated by challenging the animals intraduodenally, as outlined above, and the test interval was reduced to 16 hr. Nonetheless, the results, as summarized in Table 2, were parallel to those of the 24-hr experiment. Although the extent of translocation of organisms to mesenteric lymph nodes was generally less at this shorter interval than at 24 hr (with blood cultures only sporadically positive at 16 hr), the germ-free mice exhibited significantly more *S. typhimurium* in their lymph nodes than did their conventional counterparts. However, as seen in Table 2, this again could be construed as but a reflection of greater numbers of organisms growing intraluminally in the intestine.

In the last series of experiments, ligation of the terminal ileum was performed immediately prior to intraduodenal challenge of the animals, creating, in essence, an infection within a closed tube. As a result, the growth of *S. typhimurium* within the bowel in both groups was greater than in previous experiments, owing to the inability of the ligated bowel to discharge its content continually. Moreover, with this maneuver, as seen in Table 3, equal intraluminal growth was finally achieved in germ-free and conventional mice. With equal numbers of *S. typhimurium* thus maintained in the

bowel during the test interval, the number of organisms recovered from mesenteric lymph nodes was found to be virtually identical in the two groups of animals.

Thus, the final number of *S. typhimurium* achieved was no different in the presence or the absence of a resident flora in the ligated bowel, but was strikingly lower in the conventional animals when peristaltic emptying of the bowel was not prevented. This suggested a possible difference in the rate of propulsion of intestinal contents in germ-free and conventional mice. To test this possibility, groups of germ-free and conventional mice were fed 0.5 ml of an aqueous suspension of carmine dye via gastric tube, and then were sacrificed at intervals of 2, 4, and 6 hr

FIG. 1. Gastrointestinal tract of conventional (above) and germ-free mice fed an aqueous carmine suspension via intragastric tube 6 hr prior to sacrifice. The small intestine of the germ-free animal contains a greater residual amount of dye than does its conventional counterpart. The distended cecum, characteristic of the germ-free mouse, is evident at the left.

TABLE 3. Geometric mean counts of *Salmonella typhimurium* ETS 9 16 hr after ileal ligation and intraduodenal challenge with 10^7 organisms

Status and no. of mice	Organisms in small intestine	Organisms in mesenteric node
Germ-free (12)	9.6×10^{10}	2.0×10^2
Conventional (12)	1.1×10^{11}	4.0×10^2

after feeding. Despite the lack of precision inherent in this dye method, a consistent finding was the presence of obviously greater quantities of residual dye in the upper gastrointestinal tract, particularly the small intestine (Fig. 1), of germ-free animals than in that of conventional animals.

DISCUSSION

The results of these experiments are certainly in accord with the general principle that the presence of a resident flora influences the course of an enteric infection. The manner in which this is accomplished in the small intestine, however, was found to be somewhat different than had been anticipated at the outset of this study. Our original hypothesis was that the mucosa of the conventional animal, by virtue of its better developed lamina propria and lymphoid tissue, and of its more rapid rate of epithelial renewal (1), might prove to be a better barrier to bacterial invasion than that of the germ-free animal. The fact that the translocation of organisms across the mucosa was the same in germ-free and conventional mice, when the number of challenge organisms in the bowel was held constant during the test period, clearly demonstrates this hypothesis to be incorrect.

Instead, the present results indicate that the size of the intraluminal population of potentially invasive organisms is a prime determinant of bacterial translocation across the mucosa. Although this is true, it should be noted that alterations of rather large magnitude in the intraluminal population are required to effect much smaller changes in the extent of translocation of organisms. This is a reflection of the fact that, in each of the above experiments, as well as in previous studies with other agents (9, 12), the number of organisms actually crossing the mucosa represents only a small fraction of the total intraluminal population.

Comparison of the numbers of *S. typhimurium* within the unligated small bowel of germ-free and conventional animals leaves no doubt that the resident flora somehow exerts a controlling influence over the population of invading organisms. However, the fact that the growth of these organisms is unimpaired by the resident flora in the *ligated* small bowel of conventional animals demonstrates clearly that control is not via a direct, antibacterial action of the flora. This contrasts sharply with the action of the cecal and colonic flora, which is qualitatively and quantitatively different from that of the small intestine flora and which is known to have a direct inhibitory effect on the growth of potential pathogens (3, 13). Our results relating to the small bowel, however, agree with those of Miller and

Bohnhoff (14), who found that *S. enteritidis* is capable of multiplying in the small bowel of the mouse if peristaltic arrest is induced by morphine.

These findings are in accord with the principle that peristaltic emptying of the normal small intestine constitutes its chief defense against the buildup of large, intraluminal bacterial populations. This principle has been elegantly illustrated by the work of Dixon (4), who could find no evidence of bactericidal activity within the small intestine, but could find a clear indication of continual depletion of the bacterial population due to the normally rapid peristaltic activity. More recently, the immediate importance of this to the host has been demonstrated in the experimental models of shigellosis (6) and salmonellosis (Kent et al., *Federation Proc.* 25: 456, 1966) in the guinea pig. In both instances, pharmacologically induced impairment of intestinal motility resulted in maintenance of sufficiently large numbers of pathogenic organisms within the small intestine for a long enough time that significant penetration of the mucosa occurred.

Thus, the results of the present study lead to the conclusion that the conventional flora contributes to the control of the population of *S. typhimurium* in the small intestine primarily by stimulating peristaltic emptying. Recently, we have initiated quantitative studies of intestinal motility employing a radioactive tracer. The data thus far obtained confirm and extend the findings of the above dye study; they demonstrate that, at the end of any given experimental period up to 24 hr, a far greater proportion of an administered feeding remains in the small intestine of the germ-free animal than in that of its conventional counterpart, which is stimulated by the resident flora. Therefore, when the germ-free host is fed a challenge dose of viable bacteria, a significant fraction of that dose may remain in the small intestine over a period of many hours. Bacterial multiplication can then result in the maintenance of, if not the actual increase in, a large bacterial population to which the mucosa is exposed. This would explain not only the present results, but possibly also the findings of Formal et al. (6) and Sprinz et al. (16) that the germ-free guinea pig, unlike its conventional counterpart, is exquisitely sensitive to infection of the small intestine with *Shigella flexneri*.

The identity of the particular components of the conventional flora responsible for enhancement of intestinal motility is, at present, unknown. The site of their action is likewise obscure. Although cecal distension has long been recognized as an aspect of germ-free life, it is apparent that the small intestine and probably other portions of the gastrointestinal tract are also affected by

the presence or absence of a microbial flora. There is evidence that the neurons of Auerbach's plexus are less active metabolically in the absence of a flora (5), that there are differences in the intestinal content of pharmacologically active amines in germ-free and conventional animals (15), and that the normal flora may inactivate biochemical substances otherwise present in the cecal contents of germ-free animals (10). These findings leave no doubt that the normal microbial flora has a significant physiological impact upon the gastrointestinal tract of the host, but it is evident that the precise manner in which this impact is mediated remains to be elucidated.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-05827-02 from the National Institute of Allergy and Infectious Diseases and by a grant from The University of Michigan Cancer Research Institute.

We express appreciation to Samuel B. Formal for supplying the *S. typhimurium* used in this study, and to Eddie J. Burks for assisting in the gnotobiotic aspects of the work.

LITERATURE CITED

1. ABRAMS, G. D., H. BAUER, AND H. SPRINZ. 1963. Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germfree and conventional mice. *Lab. Invest.* **12**:355-364.
2. ABRAMS, G. D., J. E. BISHOP, H. D. APPELMAN, AND A. J. FRENCH. 1960. Development of a laboratory for germfree research in the department of pathology. *Univ. Mich. Med. Bull.* **26**:165-175.
3. 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 initiation of infection by oral inoculation. *J. Exptl. Med.* **120**:805-816.
4. DIXON, J. M. S. 1960. The fate of bacteria in the small intestine. *J. Pathol. Bacteriol.* **79**:131-140.
5. DUPONT, J. R., H. R. JERVIS, AND H. SPRINZ. 1965. Auerbach's plexus of the rat cecum in relation to the germfree state. *J. Comp. Neurol.* **125**:11-18.
6. FORMAL, S. B., G. D. ABRAMS, H. SCHNEIDER, AND H. SPRINZ. 1963. Experimental *Shigella* infections. VI. Role of the small intestine in an experimental infection in guinea pigs. *J. Bacteriol.* **85**:119-125.
7. 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.
8. FRETER, R. 1962. In vivo and in vitro antagonism of intestinal bacteria against *Shigella flexneri*. II. The inhibitory mechanism. *J. Infect. Diseases* **110**:38-46.
9. GERICHTER, C. B. 1960. The dissemination of *Salmonella typhi*, *S. paratyphi A*, and *S. paratyphi B* through the organs of the white mouse by oral infection. *J. Hyg.* **58**:307-319.
10. GORDON, H. A. 1965. A bioactive substance in the caecum of germfree animals. *Nature* **205**:571-572.
11. GORDON, H. A., AND E. BRUCKNER-KARDOSS. 1961. Effect of the normal microbial flora on intestinal surface area. *Am. J. Physiol.* **201**:175-178.
12. HILDEBRAND, G. J., AND H. WOLOCHOW. 1962. Translocation of bacteriophage across the intestinal wall of the rat. *Proc. Soc. Exptl. Biol. Med.* **109**:183-185.
13. MEYNELL, G. G. 1963. Antibacterial mechanisms of the mouse gut. II. The role of Eh and volatile fatty acids in the mouse gut. *Brit. J. Exptl. Pathol.* **44**:209-219.
14. MILLER, C. P., AND M. BOHNHOFF. 1962. A study of experimental *Salmonella* infection in the mouse. *J. Infect. Diseases* **111**:107-116.
15. PHILLIPS, A. W., H. R. NEWCOMB, J. E. SMITH, AND R. LACHAPPELLE. 1961. Serotonin in the small intestine of conventional and germfree chicks. *Nature* **192**:380.
16. SPRINZ, H., D. W. KUNDEL, G. J. DAMMIN, R. E. HOROWITZ, H. SCHNEIDER, AND S. B. FORMAL. 1961. The response of the germfree guinea pig to oral bacterial challenge with *Escherichia coli* and *Shigella flexneri*. *Am. J. Pathol.* **39**:681-695.