Interaction in the Germfree Mouse Intestine of Colicinogenic and Colicin-Sensitive Microorganisms (33773)

NORMAN S. IKARI,[1] DOLORES M. KENTON,[2] AND VIOLA MAE YOUNG[2]

Laboratory of Germfree Animal Research, National Institute of Allergy and Infectious Diseases and Microbiology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

Colicin, an extracellular antibiotic protein which is synthesized by some enteric bacteria, is lethal for colicin-sensitive enteric bacteria in *in vitro* experiments. This observation has prompted some investigators (1–4) to question whether colicin might afford a selective advantage to colicinogenic bacteria in the microbial interaction of the intestinal flora. Friedman and Halbert (5) have shown preferential intestinal establishment of antibiotic producing *Escherichia coli* when mixed cultures were inoculated orally into suckling mice. The role of colicin in maintaining the dominant flora has been inferred by results obtained by Branche *et al.* (2) and Young *et al.* (4). In the present study an attempt was made to further resolve this problem by utilizing an *in vivo* system. To afford a controlled and self-limiting gastrointestinal flora, germfree mice were inoculated, *per os*, with a dual colicinogenic colicin-sensitive bacterial system and the respective bacterial populations were determined. In addition, *in vivo* transfer of colicinogeny to the sensitive parent *E. coli* was studied.

Materials and Methods. Bacterial strains. Two strains, the first a colicinogenic slow lactose fermenting (SLF) "atypical *Citrobacter* sp.", strain CA-62, sensitive to streptomycin and producing colicin J, and the second a derivative of *E. coli* K-12 (Row), resistant to streptomycin and sensitive to colicin J, were obtained from Dr. Philip E. Hartman, Department of Biology, Johns Hopkins University, Baltimore, Md. 21218.

Media. Nutrient agar (Difco), 23 g, and nutrient broth (Difco), 8 g, were utilized with 1 and 4 gm, respectively, of NaCl added per liter. Soft agar was made with 0.7% BBL (Baltimore Biological Laboratory) agar and 0.1% NaCl added. Eosin methylene blue agar (EMB) (Difco) was the differential plating medium. Streptomycin, at a concentration of 1000 μg/ml was added to all agars when necessary.

Detection of colicinogenic (Col) factor. In vitro transfer of the Col factor was detected by the agar double-layer technique of Fredericq (1). Log phase broth cultures of colicinogenic streptomycin-sensitive CA-62 and non-colicinogenic streptomycin-resistant K-12 were mixed, incubated and plated on nutrient agar plates containing streptomycin. After 18–24-hr incubation at 37°, these plates were overlaid with the same colicin-sensitive K-12 incorporated in soft agar. An additional 18–24-hr period of incubation at 37° resulted in zones of inhibition surrounding the colicinogenic colonies. In the *in vivo* experiments, appropriate dilutions of weighed fecal samples were plated onto nutrient agar and onto EMB plates with and without streptomycin. The remaining procedure was carried out as for *in vitro* tests.

Germfree mice. White Swiss mice, NIH strain, were obtained as germfree weanlings from the Laboratory Aids Branch, NIH. They were maintained in sterile steel Reyiers tanks or in plastic isolators and fed a semi-synthetic, steam-sterilized diet, L-356 (6).

Bacterial inoculation. Eighteen-hour nutrient broth cultures of either CA-62 or *E. coli* K-12 were centrifuged and the packed viable cells resuspended to the original volume in sterile physiological saline. The suspensions were adjusted to a given concentration and introduced into sterile glass ampuls, heat-sealed and passed into the isolator via a

[1] Present address: Microbiology Section, Division of Pathology and Laboratories, D. C. General Hospital, Washington D. C. 20003.

[2] Present address: National Cancer Institute, Baltimore Cancer Research Center, U. S. Public Health Service Hospital, Baltimore, Maryland.

peracetic acid vapor lock (7). All inoculations were made *per os* by 1 or 2-ml syringes fitted with 1-in. hypodermic needles which were tipped with a small, smooth, metal, pear-shaped bulb. One-tenth ml was given per mouse. The germfree isolators generally contained 30–60 animals of mixed sexes. At 8–14 weeks of age, one-half of the animals were inoculated while the remaining half served as uninoculated controls to follow the course of cross contamination.

Results. In preliminary experiments, *in vitro* transfer of the Col factor from strain CA-62 to *E. coli* K-12 was demonstrated on nutrient agar and EMB streptomycin plates. The frequency of transfer varied from approximately 1–10% of the parental noncolicinogenic population.

When germfree mice were inoculated with these 2 strains, Col factor transfer was demonstrable at low rates of 1–2% in cultures of diluted fecal samples. The extent of Col factor transfer was not affected by allowing one or the other of the two strains to become established first in the mouse intestine followed 2 days later by the addition to the drinking water of the second strain.

Since it appeared that Col factor transfer between these two bacterial strains did occur *in vivo* as well as *in vitro*, we next considered the relative survival of these two species as the sole microbial inhabitants of the gastrointestinal tract. The extent of each bacterial strain's establishment in the intestine was investigated independently using 2 groups of 35 germfree mice with each group in separate isolators. Each group was inoculated with 2×10^7 organisms. While inoculation of the viable bacterial cells did result in generalized malaise in the majority of the animals, this adverse effect was short-lived and all affected animals recovered within 18–20-hr post inoculation. Within 24 hr, bacterial counts in the feces of these monocontaminated animals reached levels of 3 to 5.3×10^8 bacteria/0.01 g of feces (upper 2 graphs of Fig. 1). Daily samplings for the first 7 days showed that these counts remained within the limits of 2 to 8×10^8/0.01 g for either strain.

In the next experiment, 30 germfree mice were inoculated with 2×10^7 bacteria of one strain and immediately thereafter were reinoculated with equal numbers of the opposing strain. Figure 1, bottom graph, shows that although the 2 organisms were present in approximately equal numbers throughout the 7-day sampling period, *E. coli* K-12 reached

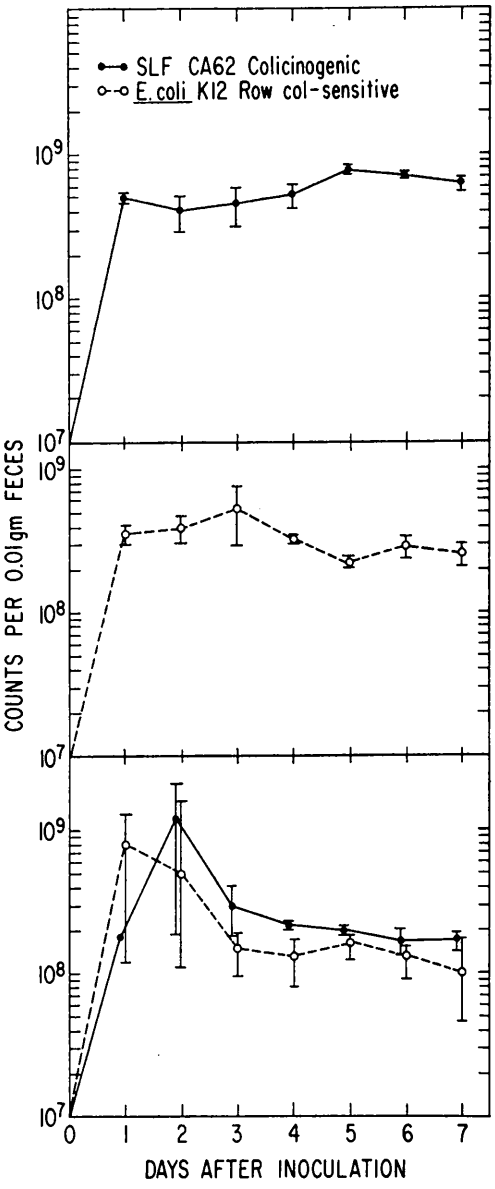

FIG. 1. Intestinal establishment of colicinogenic CA-62 and colicin-sensitive *E. coli* K-12 Row in germfree mice: vertical bars represent range of bacterial counts for each sampling point.

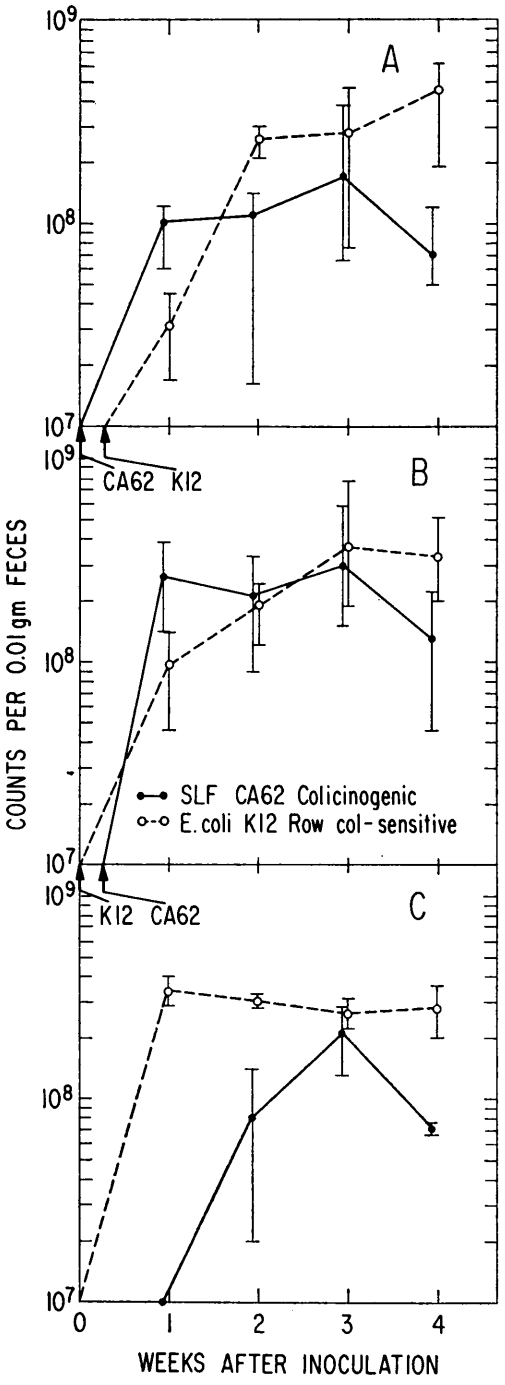

FIG. 2. Population changes of colicinogenic CA-62 and colicin-sensitive *E. coli* K-12 Row in the intestinal tract of experimentally infected germfree mice: (A) 2×10^7 CA-62, *per os*, followed by drinking water inoculation of 1×10^8 K-12; (B) 2×10^7 K-12, *per os*, followed by drinking water inoculation of 1×10^8 CA-62; and (C) uninoculated but cross-infected mice from (B).

its highest level of growth earlier than strain CA-62.

Another group of 30 germfree mice was inoculated with 2×10^7 colicinogenic CA-62 bacteria and 5 hr later, 2 ml of a broth culture of colicin-sensitive K-12 bacteria (1×10^8/ml) was introduced into the drinking water bottles (final concentration approximately 2×10^5 ml) of each inoculated jar of mice to determine if small numbers of colicin-sensitive organisms consumed over a period of time could become established as intestinal flora in the presence of a colicin producer. Fecal samples at day 4 and at additional periodic intervals showed these 2 organisms to be present in a range of 1 to 2×10^8/0.01 g of feces. Also when the experiment was terminated 5 weeks after inoculation, the colicinogenic CA-62 organisms were present in a range of 2×10^7 to 1.9×10^8/0.01 g of feces and the K-12 bacteria were in a range of 2.2 to 4.7×10^8/0.01 g of feces.

This appreciable level of colicin-sensitive K-12 bacterial counts in the presence of cells synthesizing colicin was further examined by performing population studies in separate groups of 60 germfree mice with the initial inoculum of 2×10^7 viable bacteria of either strain in one-half of each group followed by water bottle inoculation 2 days later with approximately 10 bacteria of the second strain. When the colicinogenic CA-62 was established for 2 days prior to contact with the sensitive K-12, the latter outnumbered the colicinogenic organisms (Fig. 2A). When the colicin-sensitive K-12 was the initial inoculum (Fig. 2B), a slight suppression of these bacteria was noted at the first week. However, by the fourth week postinoculation, K-12 bacteria were again predominant in the intestine. Furthermore, samplings from the 30 cross-infected uninoculated mice (Fig. 2C) in the isolator, which also contained the animals initially inoculated with K-12 bacteria (Fig. 2A), established and maintained larger numbers of colicin-sensitive K-12 than the

colicin producing strain, including the fourth week postexposure.

Discussion. The germfree animal serves as an ideal tool for the examination of an unlimited range of enteric bacterial interactions. Consequently, studies of Shigella infection (8), transfer of drug resistance (9) and multistrain colonization (11, 12), have been conducted in germfree animals. *In vivo* genetic transfer between enteric bacteria has also been performed in conventional antibiotic-treated mice (12, 13) and in newly hatched chicks (14). The transfer of colicinogeny as another form of bacterial genetic exchange was observed to take place in the animal intestine in this study.

Current interest in colicin has been centered largely upon its genetics and mode of action. Also of interest must be its role, if any, in the possible advantage for an organism producing this lethal antibiotic protein to establish and/or maintain itself in the intestine. We have approached this question by establishing a flora consisting of colicinogenic and colicin-sensitive organisms in the intestines of germfree mice. The colicinogenic CA-62 strain used in this study established and stabilized as a monocontaminant at levels comparable with the colicin-sensitive *E. coli* K-12 separately monocontaminated (Fig. 1, upper 2 graphs). Somewhat higher levels of colicinogenic CA-62 than colicin-sensitive K-12 appeared after the fourth day postinoculation and was thus maintained until the termination of the 7-day sampling period. Simultaneous residence of the colicinogenic colicin-sensitive pair (Fig. 1, bottom graph) resulted in no significant diminution of colicin-sensitive K-12 bacteria over the 7-day sampling period.

In the final set of experiments, initial tube feeding of one strain was followed 2 days later by water bottle inoculation of the other strain. In spite of the 48 hr initial advantage of colicinogenic CA-62 bacteria (Fig. 2A), colicin-sensitive *E. coli* K-12 dispersed in the drinking water resulted in the former being outnumbered by the fourth week postinoculation. Conversely, initially established colicin-sensitive K-12 (Fig. 2B) was only slightly suppressed at day 7 postinoculation, but was still present in comparable numbers in the feces at the fourth week postinoculation. In time, uninoculated animals in this isolator became naturally cross-infected as manipulative procedures necessary for isolator maintenance enhanced the dispersion of bacteria excreted from inoculated mice in adjoining jars. Figure 2C shows that both organisms established readily but colicin-sensitive K-12 was obviously not outnumbered by colicinogenic CA-62 at the end of the 4-week sampling period.

The strikingly lethal effect of CA-62 colicin upon K-12 bacteria could be seen from plating of mixed broth cultures and observation of the lytic zones in seeded agar plates. Our *in vivo* findings have not been analogous to the *in vitro* interaction which had signified an obvious dominance by the colicin producer. *In vivo* studies of colicin production (15, 16) have left no doubt that colicinogeny is a stable genetic ability. Whether strain CA-62 produces more or less colicin under either of these two conditions is not known. It appears unlikely that colicin synthesis, *per se,* would be adversely affected within the confines of the intestinal lumen as compared to growth conditions in a culture broth.

Other mechanisms which may be involved in the interplay between enteric organisms and the host and which affect our final interpretation have been proposed or studied. Meynell (17) stated that a knowledge of the rate of division, the death rate, and the rate at which inoculated organisms are excreted into the feces would clearly be of advantage. In the absence of this information, viable bacterial counts of fecal dilutions as collected in this study admittedly showed only the "net rate of change." It has been suggested that the susceptibility of colicins to the combined action of endotoxins and proteolytic enzymes would nullify any selective advantage of colicinogenic bacteria in the bowel (16, 18). Ozawa and Freter (19) concluded that availability of a fermentable carbon or energy source would influence replacement of a "resident" strain by an "invader" strain if the latter preferentially utilized this sub-

strate. In this regard, we found the colicin-sensitive *E. coli* K-12 more active in regard to various carbohydrate fermentations than the colicinogenic SLF CA-62. Other workers (17, 20) showed that volatile fatty acids and low oxidation–reduction potentials in the large intestine of normal mice exert considerable inhibitory activity against implantation of additional enteric bacteria. We are aware of the fact that examination of other similarly interacting pairs of bacterial strains in the germfree animal intestine might possibly give results covering the entire range of suppression to dominance.

It would be desirable to relate in meaningful fashion these aforementioned operational considerations and the conclusions from this study with genetic interpretations for colicinogeny in bacteria. Natural selection may have preserved plasmids which determine colicinogeny to aid in the survival and maintenance of the species so endowed. This view which was postulated by several authors (3, 21, 22) remains to be elucidated in additional ecological studies.

Summary. Synthesis of colicin, *per se,* by colicinogenic bacteria does not contribute significantly, if at all, toward a selective advantage in competition for dominance in gastrointestinal colonization. *Per os* inoculation of germfree mice with a slow lactose fermenting CA-62 colicinogenic bacteria and a colicin-sensitive *E. coli* K-12 (Row) resulted in no suppression of the latter. When fecal samples were examined over a 4-week period, the colicin-sensitive *E. coli* actually outnumbered the colicin producer.

The authors are grateful to Drs. John E. Tobie and Phillip J. Baker for reviewing the manuscript. We wish to thank Mrs. Dorothy L. Sheets, Mr. Paul C. Shade, and Mr. Preston C. Poole for their skilled technical assistance with the germfree isolators.

1. Fredericq, P., Ann. Rev. Microbiol. **11**, 7 (1957).

2. Branche, W. C., Young, V. M., Robinet, H. G., and Massey, E. D., Proc. Soc. Exptl. Biol. Med. **114**, 198 (1963).

3. Reeves, P., Bacteriol. Rev. **29**, 24 (1965).

4. Young, V. M., Branche, W. C., Kenton, D. M., and Lee, M. R., J. Pathol. Bacteriol. **92**, 303 (1966).

5. Friedman, D. R. and Halbert, S. P., J. Immunol. **84**, 11 (1960).

6. Larner, J. and Gillespie, R. E., J. Biol. Chem. **225**, 279 (1957).

7. Horton, R. E. and Hickey, J. L. S., Proc. Animal Care Panel **11**, 93 (1961).

8. Osawa, N. and Mitsuhashi, S., Japan. J. Exptl. Med. **34**, 77 (1964).

9. Kasuya, M., J. Bacteriol. **88**, 322 (1964).

10. Gibbons, R. J., Socransky, S. S., and Kapsimalis, B., J. Bacteriol. **88**, 1316 (1964).

11. Schaedler, R. W., Dubos, R., and Costello, R., J. Exptl. Med. **122**, 77 (1965).

12. Schneider, H., Formal, S. B., and Baron, L. S., J. Exptl. Med. **114**, 141 (1961).

13. Guinee, P. A. M. and van Leeuwenhoek, A., J. Microbiol. Serol. **31**, 314 (1965).

14. Walton, J. R., Nature **211**, 312 (1966).

15. Halbert, S. P. and Swick, L. S., J. Immunol. **65**, 675 (1950).

16. Braude, A. I. and Siemienski, J. S., J. Clin. Invest. **44**, 849 (1965).

17. Meynell, G. G., Brit. J. Exptl. Pathol. **44**, 197 (1963).

18. Tadd, A. D. and Hurst, A., J. Appl. Bacteriol. **24**, 222 (1961).

19. Ozawa, A. and Freter, R., J. Infect. Diseases **114**, 235 (1964).

20. Bohnhoff, M., Miller, C. P., and Martin, W. R., J. Exptl. Med. **120**, 805 (1964).

21. Brock, T. D., "Principles of Microbial Ecology," p. 129. Prentice-Hall, Englewood Cliffs, New Jersey (1966).

22. Falkow, S., Johnson, E. M., and Baron, L. S., Ann. Rev. Genet. **1**, 87 (1967).

Received Oct. 14, 1968. P.S.E.B.M., 1969, Vol. 130.