ASSOCIATION OF GERMFREE MICE WITH BACTERIA ISOLATED FROM NORMAL MICE*

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PLATE 11

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Several species of animals have been raised and made to reproduce under germfree conditions. While the animals so produced seem to have a normal life span, they exhibit histological, anatomical, and physiological characteristics which differentiate them from animals raised under conventional conditions. The very abnormalities of germfree life have thus yielded useful knowledge concerning the role played by microbial activities in the development and physiological performance of higher animals.

The availability of bacterial cultures isolated from the gastrointestinal tract of normal mice has given us the opportunity to study the consequences of associating these organisms with germfree mice. The present paper describes the results of the first long range experiments in this series. Our observations were focussed on (*a*) the fate of the bacteria in the various organs of the gastrointestinal tract following administration by feeding, and (*b*) the effect of bacteria so administered on the size of the cecum.

Materials and Methods

Five bacterial cultures were used, namely: two strains of lactobacilli (one of them being the rhizoid form); one strain of anaerobic streptococcus Group N; two strains of bacteroides; and one coliform (SLF) strain. These cultures had been recently isolated from NCS mice; their behavior in these animals is described in references 1–3. The bacteriological studies on the various organs were conducted according to the methods described in these same papers.

The germfree mice were produced by Carworth Farms Laboratory (New City, New York) and maintained in this institution until the time of the bacteriological examinations. They were then taken to our own laboratory at The Rockefeller Institute. We wish to extend to Mr. C. N. Wentworth Cumming and Mr. Dennis Baker of Carworth Laboratories our gratitude for their essential help in the design and conduct of this project.

The general program was planned as follows. The germfree mice were produced and maintained in plastic isolators of the trexler type (4 and 5). The bacterial cultures to be used for association were grown in appropriate liquid culture media (2) at The Rockefeller Institute

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and taken the next day to New City, New York. These cultures were immediately used, singly or in association as described in the text, to contaminate the food of the germfree mice in the isolators. The appropriate culture(s) were added to the food only once.

At various intervals of time (indicated for each experiment) mice were transferred to smaller isolators and were brought to The Rockefeller Institute where they were immediately autopsied. Bacteriological studies were then carried out on the stomach, small intestine, and large intestine. In a few cases, other organs were also studied bacteriologically by the same techniques (2).

Organ Localization of Various Bacterial Cultures.—Multiple groups of germfree mice, 20 per isolator, were associated with either one of the following bacterial cultures: (*a*) bacteroides (one strain); (*b*) anaerobic streptococcus of Group N; and (*c*) a mixture of two strains of lactobacillus (one of them being of the rhizoid type). Groups of animals were sacrificed 1, 3, 5, and 11 weeks after exposure to the bacteria. The numbers of bacterial colonies recovered per organ are presented in Table I.

TABLE I

*Colonies Recovered 3 Weeks after Feeding Individual Bacterial Cultures to Germfree Mice**

		Lactobacilli	Strept. (N)	Bacteroides
Stomach	Content	10^9‡	10^8	$<10^3$
	Wash§	10^3	10^6	"
	Homogenate‖	10^9	10^7	"
Small intestine	Content	10^7	10^7	"
	Wash	10^6	10^6	"
	Homogenate	10^7	10^7	"
Cecum	Content	10^9	10^8	10^{10}
	Wash	10^8	10^6	10^9
	Homogenate	10^9	10^8	10^9

* The bacterial cultures recovered from normal mice were fed to germfree mice approximately 4 weeks old. This experiment was carried out in collaboration with Mr. Wentworth Cumming and Mr. Dennis Baker of Carworth Laboratories.

‡ The figures refer to the numbers of colonies recovered per gram of organ from the animals 3 weeks after feeding cultures.

§ "Wash" refers to colonies recovered from third washing (from 1 gm of organ).

‖ "Homogenate" refers to colonies recovered per gram of washed organ homogenized in teflon grinder.

As can be seen in Table I, the numbers of bacteria recovered from the various organs, and their localization were the same as observed in NCS and other mice raised under usual conditions. Equally remarkable was the fact that colonization occurred extremely rapidly; indeed, as many colonies were recovered 1 week after feeding the culture as 3, 5, or 11 weeks later. Bacteriological tests carried out on homogenates of other organs (spleen, lungs, and

liver) failed to reveal the presence of any bacteria. It is apparent, therefore, that while the four cultures under study multiply extensively *in vivo*, they exhibit strong selective affinity for certain areas of the gastrointestinal tract. Either they do not reach other organs, or they cannot survive or multiply in them.

As indicated in Table I, and in agreement with the results reported in the preceding paper (3), the microorganisms were closely associated with the walls of the organs in which they became established.

The Behavior of Coliform Bacilli in Germfree Mice.—As reported in the first paper of this series, coliform bacilli (slow lactose fermenters) undergo a short period of extensive multiplication in young mice, then progressively decrease in numbers. Under normal conditions, they constitute but a very small percentage of the total intestinal flora of adult animals, especially in NCS mice. However, a totally different picture emerged when these bacteria were fed to germfree mice. It seems worthwhile, therefore, to consider two separate aspects of their behavior in these animals.

A culture of coliform bacilli (SLF), isolated from NCS mice, was grown in dextrose broth. It was fed to germfree mice in isolators, and also to NCS mice maintained under ordinary conditions. Animals were sacrificed 1 week and 10 weeks after feeding the culture, and the numbers of bacteria in their organs were determined by the usual techniques.

Within 24 hours after feeding, large numbers of bacilli could be isolated from the digestive tract of NCS mice. On the following day, however, this population fell precipitously and after that time it remained at the level of 10^8 bacilli per gram of large intestine, which is the level commonly observed in NCS mice. The situation was very different in the germfree mice. In these animals the coliform bacilli multiplied to a very high level in the large intestine (10^9 bacilli per gram of tissue) and also in the small intestine and the stomach (10^8 and 10^6 bacilli per gram of tissue respectively). Moreover, the coliform population remained at these high levels in all three organs throughout the 11 weeks period of the test.

There was still another difference in the behavior of the coliform bacilli in the two groups of mice. The bacilli recovered from the NCS mice retained their original biochemical characteristics, fermenting lactose only slowly when inoculated into lactose media. In contrast, a large percentage of the colonies recovered from the germfree mice fermented lactose immediately, behaving in this respect as if they had been converted into *Escherichia coli*.[1]

Other experiments were conducted to determine the effect of association of

[1] Serological analyses carried out by Dr. Rose Mushin indicate that even though these cultures had acquired the ability to ferment lactose, they had the same antigenic structure as the cultures of slow lactose fermenter isolated from the mouse colonies of The Rockefeller Institute. These findings will be described in detail in a later publication.

germfree mice with other microbial species on the behavior of coliform bacilli in these animals.

Germfree mice were associated by feeding with one strain of anaerobic streptococcus (Group N) and two different strains of lactobacilli (one of them being the rhizoid form). One week later they were fed a culture of bacteroides; then again 1 week later a culture of coliform bacilli (SLF).

Bacteriological tests carried out at weekly intervals revealed that the lactobacilli, the streptococci, and the coliform bacilli colonized the whole gastrointestinal tract, whereas organisms of the bacteroides cultures were recovered only from the large intestine where they multiplied extensively. In other words, the bacterial species behaved exactly as if they had been associated singly with the germfree animals. The only difference noted was that, contrary to what had happened in germfree mice having received only coliform bacilli, these organisms did not mutate to rapid lactose fermenters when they colonized the mouse in association with the lactobacilli, (Table II).

TABLE II

Number of Colonies Recovered 3 Weeks After Feeding Multiple Bacterial Cultures to Germfree Mice‡*

	Lactobacilli	Strept. (N)	Bacteroides	Coliforms (SLF) and Enterococci
Stomach	10^9§	10^8	$<10^3$	10^6
Small intestine	10^8	10^7	"	10^8
Large intestine	10^9	10^8	10^9	10^9

* The same results were obtained 4 months after feeding the cultures, and also in similar tests on the offspring of these animals.

‡ The bacterial cultures recovered from normal mice were fed to germfree mice approximately 4 weeks old. This experiment was carried out in collaboration with Mr. Wentworth Cumming and Mr. Dennis Baker of Carworth Laboratories.

§ The figures indicate the approximate numbers of colonies recovered on selective media per gram of organ homogenate.

After these observations had been made, a culture of enterococci was introduced into the isolator. Like the coliform bacilli, the enterococci multiplied rapidly and invaded all the organs of the gastrointestinal tract.

Table II presents the picture of the bacterial population in the various organs after all the bacterial cultures had been introduced. The aspect of the bacterial population dynamics which deserves most emphasis, however, is that the coliform bacilli and enterococci remained extremely numerous throughout the period of observation (4 months) and furthermore that these organisms continued to colonize the stomach and the small intestine. In these respects, therefore, the germfree mice differed profoundly from mice raised under ordinary

conditions, since extensive invasion of the gastrointestinal tract by enterococci and coliform bacilli is extremely transient in the latter animals.

At the end of the experiment, fresh fecal material from NCS mice was introduced into the isolator containing the germfree animals associated with the different bacterial cultures. Within 2 days, the numbers of enterococci and coliform bacilli decreased precipitously, soon reaching the level of 10^3 per gram of tissue or of fecal material characteristic of NCS mice. It appeared, therefore, as if the NCS mice had contributed a transmissible agent, as yet unidentified, capable of affecting the composition of the gastrointestinal flora.

While the phenomena described above are far too complex, and insufficiently analyzed, to justify any extensive discussion of their mechanisms, they suggest nevertheless two remarks which may be of relevance to the physiology of the gastrointestinal tract. As we have seen, the invasion of the whole tract by enterococci and coliform bacilli persisted unabated for several months in germfree mice. Since these animals are known to be capable of producing a normal immunological response to antigenic stimuli, it would appear that ordinary immune reactions do not play a significant role in controlling the microbial population in the gastrointestinal tract. On the other hand, the enterococcal and coliform populations were sharply reduced as soon as the germfree mice came in contact with fecal material from normal pathogen-free NCS mice; it seems possible, therefore, that this change was brought about by a microbial agent which in some way can exert an antagonistic action against other microorganisms. The foregoing experiments provide convenient laboratory models for the isolation of this agent, and experiments to this end are now being organized.

The Morphogenetic Effects of the Bacterial Flora.—Among the many abnormalities of germfree animals some of the most striking are those which interfere with the normal histological development of the intestinal epithelium, and which bring about a gross enlargement of the cecum. It has been known for several years that these abnormalities are rapidly corrected when the germfree animals are associated with some components of the gastrointestinal flora. (5–9)

In the experiments reported above, association of germfree mice with lactobacilli and anaerobic streptococci (Group N) corrected the cecal enlargement only slowly and imperfectly. The beneficial effect was more rapid, and more striking, when the animals were associated with bacteria of the bacteroides group.

In another experiment, germfree animals associated with lactobacilli, anaerobic streptococci (Group N), bacteroides, enterococci, and coliform bacilli were allowed to breed in an isolator. The bacterial population of the young animals thus obtained was determined on several occasions and was found to be identical to that of their parents and therefore different from that of ordinary mice (Table II). However, their intestine and cecum appeared entirely normal, with regard to both histological structure, shape, and size (Fig. 1).

SUMMARY

Germfree mice were given food contaminated with pure cultures of various bacterial species isolated from ordinary healthy mice. The cultures were given singly, or in association, or consecutively at weekly intervals.

Whatever the technique of administration, the lactobacilli and anaerobic streptococci immediately established themselves throughout the gastrointestinal tract, and became closely associated with the walls of the organs. In contrast, the organisms of the bacteroides group were found in large numbers only in the large intestine.

Within a week after exposure, the populations of these three bacterial species reached levels similar to those found in ordinary mice. They remained at these characteristic levels throughout the period of observation (several months). Their presence resulted in a progressive decrease in the size of the cecum which eventually became normal in gross appearance.

Coliform bacilli multiplied extensively and persisted at high levels in all parts of the gastrointestinal tract of germfree mice, even after these had become colonized with lactobacilli, anaerobic streptococci and bacteroides. However, the coliform population fell precipitously within a few days after the animals were fed the intestinal contents of healthy pathogen-free mice.

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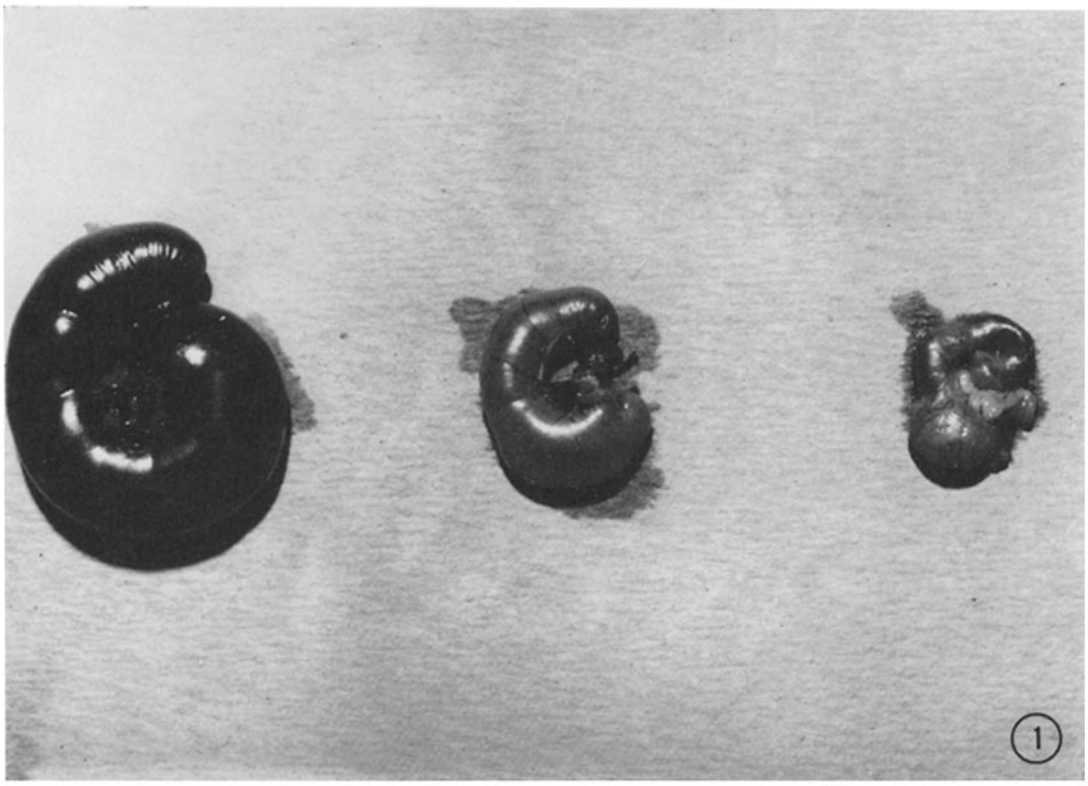
EXPLANATION OF PLATE 11

FIG. 1. Left, cecum of germfree mouse (19 per cent of total body weight); right, cecum of conventional mouse (1.6 per cent of total body weight); middle, cecum of germfree mouse reassociated with lactobacilli and other bacilli (4.5 per cent of total body weight). × 3.

Pictures taken in collaboration with Mr. Dennis Baker of Carworth Laboratories.



(Schaedler *et al.*: Germfree mice and bacteria)