INFECTION AND IMMUNITY, Oct. 1970, p. 376-386
Vol. 2, No. 4

Printed in U.S.A.

Efficiency of Various Intestinal Bacteria in Assuming Normal Functions of Enteric Flora After Association with Germ-Free Mice

SALAM A. SYED, GERALD D. ABRAMS, AND ROLF FRETER
Departments of Microbiology and Pathology, The University of Michigan, Ann Arbor, Michigan 48104

Received for publication 20 March 1970

Strictly anaerobic and facultatively anaerobic bacteria were isolated from the intestinal tract of normal mice. Germ-free mice were associated with mixtures of varying complexity of pure cultures of these bacteria. The development of normal features in these animals was then determined on the basis of the following criteria: (i) size of the cecum, (ii) size of the *Escherichia coli* population in the cecum, (iii) histology of the intestinal tract, and (iv) development of a mucosa-associated flora in stomach and large intestine. Germ-free mice contaminated with cecal contents from conventional mice were used as controls to establish normal values for these parameters. Some strictly anaerobic bacteria could be implanted into germ-free mice only after prior implantation of an *E. coli* strain. *E. coli* was found in large numbers in stomach and cecum of mice monocontaminated with this organism. Use of restraining devices indicated that the *E. coli* population in the stomach was maintained by coprophagy and did not contribute significantly to the size of the cecal population. A mixture of 50 strictly anaerobic bacteria plus 80 facultative anaerobes rendered recipient animals normal with respect to the criteria tested. Other, less complex bacterial mixtures reduced the cecal size and the intestinal *E. coli* population to levels intermediate between those found in normal and germ-free mice. With all bacterial mixtures tested, the intestinal *E. coli* population decreased, if at all, within a period of about 10 days after introduction of other bacteria, and remained stable thereafter. This suggests that the intestinal *E. coli* population is controlled by a mechanism which reduces population size without affecting the growth rate.

It is well known that the normal enteric flora of the mammalian body contributes significantly to the host's resistance to infectious diseases. Furthermore, changes in the composition of this flora are often associated with (and possibly may be the cause of) diseases such as nonspecific diarrheas, malabsorption syndromes, etc. (3). It would therefore be highly desirable to have some means of controlling the composition of the enteric flora so as to establish a microbial population of maximal benefit to the host. Unfortunately, this is not possible at the present time, because the ecology of mammalian intestinal flora remains one of the poorly understood fields in microbiology. As pointed out in earlier publications (2, 15), one of the primary reasons for this state of affairs is the inadequacy of conventional culture methods for the isolation of the strictly anaerobic bacteria, i.e., of those microorganisms which form the predominant flora of the large intestine.

As a necessary preliminary to a study of the ecological control mechanisms which determine the composition of normal enteric flora, we described (2) an anaerobic glove-box culture technique which allows the recovery of a significant fraction (20 to 50% of total microscopic counts) of the cecal flora of the mouse. Although this constitutes a considerable improvement over conventional anaerobic culture methods (such as the anaerobic jar technique), one cannot disregard the fact that 50 to 80% of the total bacterial count is still not cultivated by our method. This may mean that a number of bacterial species cannot be cultivated in the glove box. Alternatively, a substantial fraction of the cecal bacteria may be nonviable, which, in turn, would imply that the culture method is sufficient for cultivating at least those bacteria which predominate in the intestinal population.

In the studies described below, the above question was investigated by testing whether

bacteria isolated from the normal mouse intestine would form a "normal" flora when implanted into germ-free mice. If this were possible, one could be confident that the bacterial cultures involved include at least those species which are most important in the control of the ecological balance of the intestinal tract. It would then be possible to proceed with a study of the mechanisms of bacterial interactions in the intestine. Similar attempts to "normalize" germ-free mice have been made in the past by others (e.g., 4, 7, 9). To our knowledge, all of these attempts have been unsuccessful in that a completely "normal" flora could be implanted into germ-free or streptomycin-treated mice only by feeding fecal material. Introduction of mixtures of bacteria isolated in pure culture from normal mice only partially restored the animals to their normal state.

Much of the significance of the present work depends on the criteria used for describing an experimental animal as "normal." It is obviously desirable to test for a variety of parameters which may be assumed to be mediated by a variety of different mechanisms. On the other hand, it is necessary to keep the required technical efforts at a manageable level. In view of these considerations, the following parameters were selected: (i) the reduction of the population in the cecum of an *Escherichia coli* strain to normal levels [the strain selected, *E. coli* C25, had been shown in earlier studies to suppress invading pathogens such as *Shigella* and *Vibrio* (5)]; (ii) reduction in size of the typically enlarged cecum of the germ-free mouse to normal levels; (iii) development of normal histological features in the gastrointestinal tract; and (iv) the development of a mucosa-associated layer of bacteria in the stomach and large intestine, as described by Savage et al. (13), Plaut et al. (11), and others. In accordance with common practice in germ-free research (7), ex-germ-free animals, contaminated with cecal homogenate from normal mice, were used as normal standards for the above parameters.

The experiments reported below define the necessary conditions for the implantation of strictly anaerobic bacteria into germ-free mice and describe a collection of pure cultures of bacteria which, when implanted into germ-free mice, will render the animals normal with respect to all criteria tested. The data presented also allow some preliminary conclusions as to the mechanisms involved in the control of the *E. coli* population by the other elements of the normal enteric flora.

MATERIALS AND METHODS

Mice. Bacteria of the normal flora were isolated from mice of strain BALB/wm, maintained in this department by William Murphy. Normal cecum homogenates were also prepared from organs of these mice. The germ-free mice used were Charles River strain CD-1. These were maintained in Trexler-type flexible vinyl isolators, sterilized with peracetic acid. Autoclaved powdered Lobund diet L-356 was fed in all experiments, except that shown in Table 2, in which the mice received pelleted sterile diet obtained from Charles River Mouse Farms. (This accounts for the relatively high *E. coli* population observed in that experiment.) Plastic cages containing autoclaved wood shavings were used inside the germ-free isolators.

Microorganisms. Bacteria were isolated from normal mice in an anaerobic glove box. Enriched Trypticase Soy Agar with palladium black overlay, as described in an earlier publication (2), was used for the isolation of strict anaerobes. Facultative anaerobes were isolated on the same medium. In addition, blood-agar and L B S Medium (BBL) were used for isolating facultative anaerobes and microaerophilic lactobacilli. Plates for the isolation of the latter organisms were incubated inside the anaerobic glove box as well as aerobically in a conventional incubator.

The taxonomy of anaerobic bacteria is still "in a state of flux" (14). As pointed out recently by Moore (10), most of the bacterial population of the intestine of man and animals "has not been thoroughly characterized." We can certainly confirm this statement. All strains of anaerobic bacteria used in the present study have been characterized according to most of the criteria published recently by Smith and Holdeman (14), but many strains do not fit into the taxonomic schemes proposed by these authors, by Prevot and Fredette (12), or by the Virginia Polytechnic Institute Anaerobe Laboratory (16). Since the taxonomic problem of anaerobes is pursued vigorously by other workers, no attempt has been made at this time to assign species names to the bacteria used. The anaerobic bacteria were of different morphological types, ranging from short to very long filamentous rods, with rounded or tapered ends. Some had vacuoles (spores?) and were resistant to heating at 70 C for 10 min. The Gram reaction was generally negative, but many strains showed gram-positive inclusions of various sizes and for this reason would perhaps be considered gram-positive by other workers. All morphological types of bacteria which can be seen in Gram-stained smears from ceca of normal mice were represented among the cultures of anaerobes used in this study. The facultative anaerobes used in this study included enterobacteria, such as *Aerobacter*, *Proteus*, and *Pseudomonas* species, *Staphylococcus albus*, *Streptococcus faecalis*, *Lactobacillus*, and bacteria resembling the *Actinomyces-Bifidobacterium* group (14).

The strain of *E. coli* C25 was the same one used in earlier studies in this laboratory (5).

Inoculation of animals. Suspensions of bacteria were prepared inside the anaerobic chamber. These were transferred into ampoules which were then stoppered, removed from the glove box, and sealed without contamination by atmospheric oxygen. After introduction into the germ-free isolators, one ampoule was opened just before inoculation of each animal. The mice were injected with 0.5 ml of suspension via

the rectum, through a 0.060-inch (outer diameter) polyethylene tube (Adams Intramedic tubing size PE100). Preliminary tests had shown that this procedure results in the introduction of some material directly into the cecum. Rectal inoculation was used in all experiments described in the present paper because this procedure had been found superior to oral inoculation by R. W. Schaedler (*personal communication*). Later experiments, not reported here, suggest that this precaution may not have been necessary with the strict anaerobes used in the present study. These microorganisms may be established in germ-free mice via the drinking water. Exposure to the oxygen in the drinking water results in a dramatic drop in the bacterial (plate) count, but a sufficient number of bacteria appear to remain viable long enough to initiate growth in the mouse intestine.

Quantitative bacterial cultures. Organs to be studied were homogenized in 100 ml of Trypticase Soy Broth in a Waring Blendor. Serial dilutions of the homogenates were plated on the surface of Deoxycholate Agar (BBL) containing 1 mg of streptomycin per ml. This medium allows the growth of *E. coli* C25 while suppressing all other bacteria used in this study.

Microscopic bacterial counts. Counts were performed on organ homogenates prepared as above for quantitative cultures. A Petroff-Hausser chamber was used as described earlier (15).

Restraining devices to prevent coprophagy. Tubes of acrylic plastic 1 inch in inner diameter by 3.25 inches long (2.5 by 8.25 cm), which contained the animals comfortably but were sufficiently narrow to keep the mice from turning around, were used. An opening [0.75 by 0.75 inch (1.9 by 1.9 cm)] in the anal region allowed fecal material to be dropped. The front of the tube led into a food hopper, which also permitted access to a drinking tube leading from a water bottle overhead.

Histopathological techniques. Specimens of the gastrointestinal tract were fixed in 10% Formalin, embedded in paraffin, sectioned at 5 μm, and routinely stained with hematoxylin and eosin for histopathological examination. Segments of small and large intestine were processed without opening or disturbing the included contents, whereas portions of stomach and cecum were fixed after opening, but with adherent contents disturbed as little as possible. Some material was also frozen in methylcellulose solution (13), but, as there appeared to be no particular advantage to the method, the observations recorded below were derived entirely from paraffin-embedded material.

RESULTS

Table 1 shows the results obtained in attempts to implant a gram-negative anaerobic bacterium into germ-free mice, into ex-germ-free mice which had been exposed to *E. coli* C25 plus a strain of *Lactobacillus* 3 days previously, into ex-germ-free mice which had been exposed to lactobacilli (same strain used above) 3 days previously, and into germ-free mice, the inoculum of anaerobes being mixed with the same *Lactobacillus* and *E. coli* strains used above. The mice were killed 5 or more days after inoculation, and the presence of anaerobic bacteria was determined by anaerobic culture and microscopic observation of cecal homogenates. Presence of the anaerobes at 5 or more days after inoculation was considered evidence of successful implantation. In all instances, the *E. coli* or the lactobacilli, or both, grew to high levels after the mice were exposed to them in their drinking water. The results in Table 1 show that the anaerobes could be implanted only in animals carrying a previously established flora of *E. coli*. The *Lactobacillus* strain used was ineffective, as was simultaneous inoculation of the anaerobes in mixed culture with *E. coli*. Consequently, all subsequent experiments involving implantation of anaerobes were carried out after *E. coli* had been established in the mice for at least 2 days.

TABLE 1. *Implantation of a strictly anaerobic, gram-negative, rod-shaped bacterium into the intestinal tract (cecum) of germ-free and gnotobiotic mice*

Inoculum	Recipient	Implantation of anaerobes
Anaerobes	Germ-free	0/17[a]
Anaerobes	Carrying anaerobic lactobacillus plus *E. coli* C25	46/57
Anaerobes	Carrying anaerobic lactobacillus only	0/4
Anaerobes plus *E. coli* C25, plus anaerobic lactobacillus	Germ-free	0/11

[a] Number of mice in which anerobes became established/total number of animals inoculated.

TABLE 2. *Recovery of E. coli C25 on streptomycin-containing Deoxycholate Agar from mice monocontaminated for 91 days*

Mouse no.	No. of *E. coli* cells per cecum cultured	Microscopic counts of *E. coli* cells per cecum
36/5	1.8×10^{10}	1.97×10^{10}
36/6	2.0×10^{10}	1.46×10^{10}
36/7	1.3×10^{10}	1.8×10^{10}
36/8	1.4×10^{10}	2.0×10^{10}
36/9	2.2×10^{10}	3.0×10^{10}
36/10	2.9×10^{10}	2.2×10^{10}
36/11	1.8×10^{10}	2.1×10^{10}

One important feature in the experiments described below is the determination of the *E. coli* population in the cecum. To facilitate the

recovery of this organism, the streptomycin-resistant strain C25 was used, because it could be recovered quantitatively on streptomycin-containing Deoxycholate Agar, a medium which did not support the growth of any other bacterium used in these studies. To investigate the efficiency of the above culture medium in recovering this strain, germ-free mice were monocontaminated with *E. coli* C25. After 91 days, the mice were killed and their ceca were homogenized and cultured in the usual manner. As may be seen in Table 2, there was close agreement between the microscopic and viable counts within the limits of error to be expected with the methods employed. This indicates that *E. coli* could be recovered quantitatively by viable count from the mouse cecum and that there was no significant overgrowth of streptomycin-sensitive mutants under the conditions of this experiment.

It is known that normal mice may harbor a sizable bacterial population in their stomach (13). Preliminary experiments in the present study had also shown that mice monocontaminated with *E. coli* C25 harbor large numbers of this microorganism in the stomach, but not in the jejunum or upper ileum. Since subsequent experiments are to be concerned with the *E. coli* population in the cecum, it was necessary to determine the degree to which *E. coli* from the stomach contributes to the bacterial flora in the cecum. Germ-free mice were therefore monocontaminated with *E. coli* C25, and 2 days later were placed into restraining tubes to prevent coprophagy, as described under Materials and Methods. Quantitative cultures were made 6 to 10 days thereafter of homogenates from stomach or cecum.

The results (Table 3) show that all mice kept in restraining devices for more than 6 days had significantly reduced *E. coli* counts in their stomachs. Most likely, this reduction occurred as late as the 6th day, since two mice (in experiment 1) had normal counts in the stomach and two other animals (in experiment 3) showed less than normal counts at this time. The reduction in *E. coli* counts in the stomach after the 6th day was consistently in the order of 1,000-fold and is, therefore, statistically highly significant. In contrast to these findings in the stomach, there was no effect of the restraining devices on the *E. coli* population of the cecum. All mice kept in restraining devices had normal cecal *E. coli* counts which were independent of the size of the *E. coli* population in the stomach. (Additional counts of cecal *E. coli* in unrestrained, monocontaminated mice are shown in Table 4.) One must, therefore, conclude that *E. coli* multiplied in the stomach at a rate which was insufficient to maintain a large

TABLE 3. *Effect of a restraining device which prevents coprophagy on the viable counts of E. coli in stomach and cecum of monocontaminated mice*

Expt no.	Mice in restraining devices		Unrestrained control mice (kept in regular cages)	
	Stomach	Cecum	Stomach	Cecum
1	(6)[a] 6,000 × 10⁵[b]	Not done	(6) 6,300 × 10⁵	Not done
	(6) 4,600 × 10⁵	Not done	(6) 19,200 × 10⁵	Not done
	(10) <0.01 × 10⁵	Not done	(10) 18,500 × 10⁵	Not done
	(10) 0.01 × 10⁵	Not done	(10) 0.35 × 10⁵	Not done
			(10) 6,700 × 10⁵	Not done
			(10) 11,600 × 10⁵	Not done
2	(7) 10 × 10⁵	Not done	(7) 12,000 × 10⁵	Not done
	(7) 17 × 10⁵	Not done	(7) 19,000 × 10⁵	Not done
	(7) 12 × 10⁵	Not done	(9) 11,000 × 10⁵	(9) 46 × 10⁸
	(9) 1.0 × 10⁵	(9) 90 × 10⁸	(9) 4,500 × 10⁵	(9) 60 × 10⁸
	(9) 0.5 × 10⁵	(9) 110 × 10⁸	(9) 4,300 × 10⁵	(9) 50 × 10⁸
	(9) 0.15 × 10⁵	(9) 60 × 10⁸	(10) 3,950 × 10⁵	(10) 90 × 10⁸
3	(6) 0.15 × 10⁵	(6) 60 × 10⁸	Not done	Not done
	(6) 200 × 10⁵	(6) 34 × 10⁸	Not done	Not done
	(9) <0.01 × 10⁵	(9) 40 × 10⁸	Not done	Not done
	(9) 0.9 × 10⁵	(9) 80 × 10⁸	Not done	Not done

[a] Number of days the animal was kept restrained prior to culture or, in the case of the control mice, the number of days that the corresponding experimental animals had been restrained on the day of culture.

[b] Figures indicate viable (colony) counts of *E. coli* per one whole organ. Each figure represents one mouse.

TABLE 4. *Number of E. coli C25 cells in the cecum of mice associated with different types of intestinal flora*

Expt no.	Flora implanted	No. of *E. coli* cells per cecum[a]					
		1–4 days[b]	5–8 days	9–12 days	13–16 days	17–21 days	26–31 days
1	*E. coli* only	111×10^8 44×10^8 30×10^8	90×10^8 38×10^8 25×10^8	60×10^8 30×10^8 20×10^8	65×10^8 46×10^8 37×10^8	60×10^8 34×10^8	60×10^8 50×10^8 33×10^8
2	*E. coli* plus one strain of *Lactobacillus*	41×10^8 38×10^8 30×10^8	17×10^8 15×10^8	40×10^8 20×10^8	45×10^8	20×10^8	14×10^8 28×10^8
3	As in 2 plus one strain each of enterococcus, *Lactobacillus*, and *Candida*, plus 4 morphologically different strains of gram-negative anaerobes			100×10^8 80×10^8	50×10^8 80×10^8 40×10^8 5×10^8	45×10^8 30×10^8 20×10^8	20×10^8
4	As in 3 plus two additional strains of gram-negative anaerobes with fusiform morphology	30×10^8 20×10^8	12×10^8 12×10^8	5×10^8 5×10^8 3×10^8 3×10^8	5×10^8 2×10^8 2×10^8		
5	As in 4 plus 30 additional strains of gram-negative anaerobes			1.9×10^8 1.6×10^8 1.0×10^8 0.9×10^8 0.6×10^8	0.97×10^8 0.88×10^8 0.87×10^8 0.70×10^8 0.50×10^8 0.40×10^8		1.0×10^8 0.76×10^8 0.70×10^8 0.56×10^8
6	Cecal homogenate from normal mouse	50×10^8 50×10^8	Median = 0.095×10^8 (same data as shown in Table 6)				

[a] Each figure represents one mouse. Counts are viable (colony) counts on Desoxycholate Agar containing streptomycin.

[b] Days after implantation of the flora. In experiments 2–6, *E. coli* was implanted at least 2 days prior to introduction of the other bacteria. The *E. coli* population on day zero of these experiments was therefore in the order of 10^9 to 10^{10} per cecum, i.e., similar to that shown in experiment 1.

population in the absence of corpophagy, and that the *E. coli* population in the cecum was not derived from the stomach to any significant degree. Since there was no large *E. coli* population in the jejunum and upper ileum of monocontaminated unrestrained mice, one must conclude that the *E. coli* population of the cecum is largely derived from local multiplication of this organism in the cecum and perhaps the lower ileum.

The main part of the present study consisted of experiments in which germfree mice were associated with mixtures of microorganisms of various complexity. Six representative experiments are shown in Table 4. In each instance, *E. coli* C25 had been established first in germfree mice, followed 2 days later by the flora described in Table 4. As may be seen, when *E. coli* grew as a monocontaminant, it established a population ranging from 20×10^8 to 111×10^8 bacteria per cecum. In animals contaminated with cecal homogenate from a normal mouse (i.e., in our "normal" standard), this *E. coli* population was reduced by a factor of about 1,000 (experiment 6). Association with known bacteria resulted in *E. coli* populations which were either unaffected (experiments 2 and 3) or reduced by factors of about 10 (experiment 4) or 100. It should be noted that in all instances tested (including the data in Tables 5–7) the *E. coli* population adjusted, if at all, within a period of about 10 days after introduction of other bacteria and remained stable thereafter. Cecal size was not determined exactly in the experiments shown in Table 4, but inspection revealed that the ceca of mice in experiments 1 to 5 were consistently larger than those of the controls (experiment 6).

In a final series of experiments, a mixture of 80 facultative anaerobes plus 50 strictly anaerobic gram-negative bacteria were tested in the usual manner for their effect on germ-free mice. The

results of one representative experiment are shown in Table 5. These data should be compared with those of Table 6 for "normalized" controls (i.e., animals fed cecal homogenate from normal mice). Values for cecal size of germ-free animals ranged from 5 to 10% of body weight.

As may be seen in Tables 5 and 6, the complex mixture of bacteria used did reduce the *E. coli* population and cecal size to levels which were within the normal range. A further control experiment indicated that the 80 facultative anaerobes used in the above experiments (Table 5) did not, by themselves, render recipient germ-free mice normal.

Table 7 shows data for mice contaminated with homogenates from stomach and jejunum of normal mice. As may be seen, the bacteria in these homogenates did not reduce the cecal

TABLE 5. *Effect of introducing 50 strains of gram-negative strict anaerobes plus 80 facultative anaerobes on size of cecum and E. coli population in gnotobiotic mice*

Mouse no.	Days after introducing flora	Cecum (% of body wt)	No. of *E. coli* cells per cecum
33.1	5	1.7	41×10^6
33.2	5	1.5	29×10^6
33.3	5	1.3	10×10^6
33.4	5	1.8	8.5×10^6
33.5	10	1.9	16×10^6
33.6	10	1.7	16×10^6
33.7	10	1.3	13×10^6
33.8	10	—	6.0×10^6
34B1	12	1.6	8.4×10^6
34B3	12	0.9	6.7×10^6
34B4	12	1.3	0.7×10^6
33.9	16	1.6	3.1×10^6
33.10	16	1.3	12×10^6
33.11	16	1.3	11×10^6
33.12	16	1.3	3.8×10^6
34B5	19	1.1	62×10^6
34B6	19	0.9	6.0×10^6
34B7	19	1.6	45×10^6
33.13	27	1.7	7.0×10^6
33.14	27	1.5	5.0×10^6
33.15	27	1.6	4.0×10^6
33.16	27	1.3	4.9×10^6
33.17	60	1.8	8.3×10^6
33.18	60	1.0	6.4×10^6
33.19	60	0.7	5.4×10^6
33.20	60	0.9	2.8×10^6
Median		1.3	7.6×10^6

TABLE 6. *Effect of feeding a homogenate of normal mouse cecum on size of cecum and E. coli population in ex-germ-free mice*

Mouse no.	Day after introducing flora	Cecum (% of body wt)	No. of *E. coli* cells per cecum
32.1	5	1.1	20×10^6
32.2	5	1.5	12×10^6
32.3	5	1.2	10×10^6
32.4	5	1.4	76×10^6
32.5	11	1.7	12×10^6
32.6	11	1.6	3.5×10^6
32.7	11	1.4	0.02×10^6
32.8	11	1.3	110×10^6
32.9	15	1.7	52×10^6
32.10	15	1.0	50×10^6
32.11	15	0.6	11×10^6
32.12	15	0.7	9.7×10^6
32.13	20	0.9	4.1×10^6
32.14	20	1.1	6.2×10^6
32.15	20		4.1×10^6
32.16	20	0.7	6.8×10^6
32.17	25	0.7	3.0×10^6
32.18	25	0.7	2.3×10^6
32.19	25	1.0	63×10^6
32.20	25	1.5	6.5×10^6
32.21	60	0.5	0.8×10^6
32.22	60	1.1	72×10^6
32.23	60	1.4	11×10^6
32.24	60	1.2	9.4×10^6
32.25	60	0.8	8.0×10^6
32.26	60	0.5	2.0×10^6
Median		1.1	9.5×10^6

E. coli population or cecal size to normal levels, thus indicating that the cecal flora was necessary for this effect.

Histopathological studies. Histopathological studies were carried out on the following groups of animals: (group 1) germ-free mice, (group 2) mice monocontaminated with *E. coli* C25 for 30 days, (group 3) mice contaminated with cecal homogenate from normal animals for 30 days (taken from the experiment shown in Table 6), (group 4) mice contaminated with a complex mixture of bacterial isolates for 30 days (taken from the experiment shown in Table 5). Four to six mice from each group were studied.

Stomachs. The histology of the gastric wall per se did not vary significantly from group to group. In all animals there were a few scattered inflammatory cells in the submucosa, this population being slightly more dense in the animals of groups 3 and 4 than in the *E. coli*-monoasso-

TABLE 7. *Effect of feeding a homogenate of normal mouse stomach and jejunum on size of cecum and E. coli population in ex-germ-free mice*

Mouse no.	Day after introducing flora	Cecum (% of body wt)	No. of *E. coli* cells per cecum
31.1	5	2.6	100×10^6
31.2	5	2.6	$3,800 \times 10^6$
31.3	5	2.8	$3,900 \times 10^6$
31.4	11	3.2	740×10^6
31.5	11	2.7	430×10^6
31.6	11	3.3	710×10^6
31.7	16	1.1	$2,000 \times 10^6$
31.8	16	3.3	850×10^6
31.9	16	3.3	540×10^6
31.10	30	3.3	910×10^6
31.11	30	2.7	280×10^6
Median		2.8	740×10^6

ciated or the germ-free animals (groups 1 and 2). In both groups 3 and 4, a dense bacterial population was intimately associated with the superficial, keratinized layers of the squamous mucosa (Fig. 1). In contrast, only rare individual bacteria were seen in the *E. coli*-monoassociated animals, no layer being formed. The keratinized layer itself appeared slightly more compact in the germ-free animals (group 1) than in members of the other three groups.

Small intestine. As expected, the mucosa of the germ-free small intestine contrasted sharply with that of the "conventional" (group 3) animals, particularly with regard to the greater development and cellularity of the lamina propria in the latter group. A similar degree of development of the mucosa was seen in group 4. In contrast, the mucosa of the animals monoassociated with *E. coli* (group 2) could not be distinguished readily from that of the germ-free mice (Fig. 2).

Cecum and colon. The cecal mucosa in germ-free animals was thin and somewhat villous, with a sparse leukocyte population in the lamina propria. In "conventional" animals (group 3), and in animals harboring the complex mixture (group 4), the mucosa was thicker and less villous, and had a greater population of inflammatory cells in the lamina propria. Monoassociation with *E. coli* (group 2) failed to alter significantly the appearance of the mucosa as compared with the germ-free state. Parallel results were obtained for the colon (Fig. 3).

Dense layers of bacteria lined the epithelial surfaces of cecum and colon, apparently in the mucous layer, in "conventional" animals (group 3). With artifacts of fixation and sectioning, it was difficult to judge the continuity of this striking layer of bacteria, but it appeared somewhat more complete in colon than in cecum. The bacterial layers in group 4 were indistinguishable histologically from those in group 3. No discrete layers of bacteria could be identified in the *E. coli*-monoassociated animals of group 2 (Fig. 4).

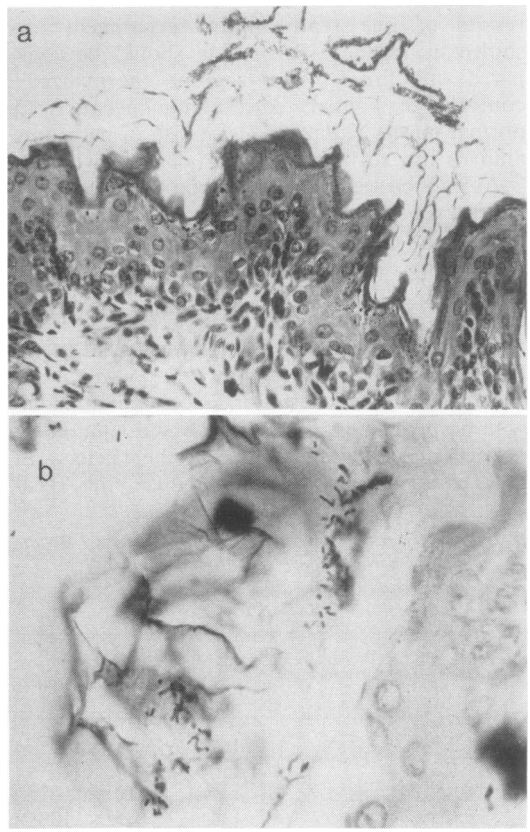

FIG. 1a. *Section of nonglandular gastric mucosa of a mouse harboring a defined flora (group 4). The granular appearance of the loose, uppermost epithelial layers is due to the presence of bacteria intimately associated with the epithelium. Hematoxylin and eosin, × 280.*

FIG. 1b. *Section of nonglandular gastric mucosa of a mouse of group 4. Numerous bacteria are adherent to superficial squamae. Methylene blue, × 685.*

In summary, then, the animals in group 4 resembled the "normal" controls (group 3) with respect to all histological features examined. In contrast, animals monoassociated with *E. coli* C25 histologically resembled germ-free mice.

DISCUSSION

The data presented indicate that it is possible with the improved culture methods used in the present study to isolate those intestinal bacteria

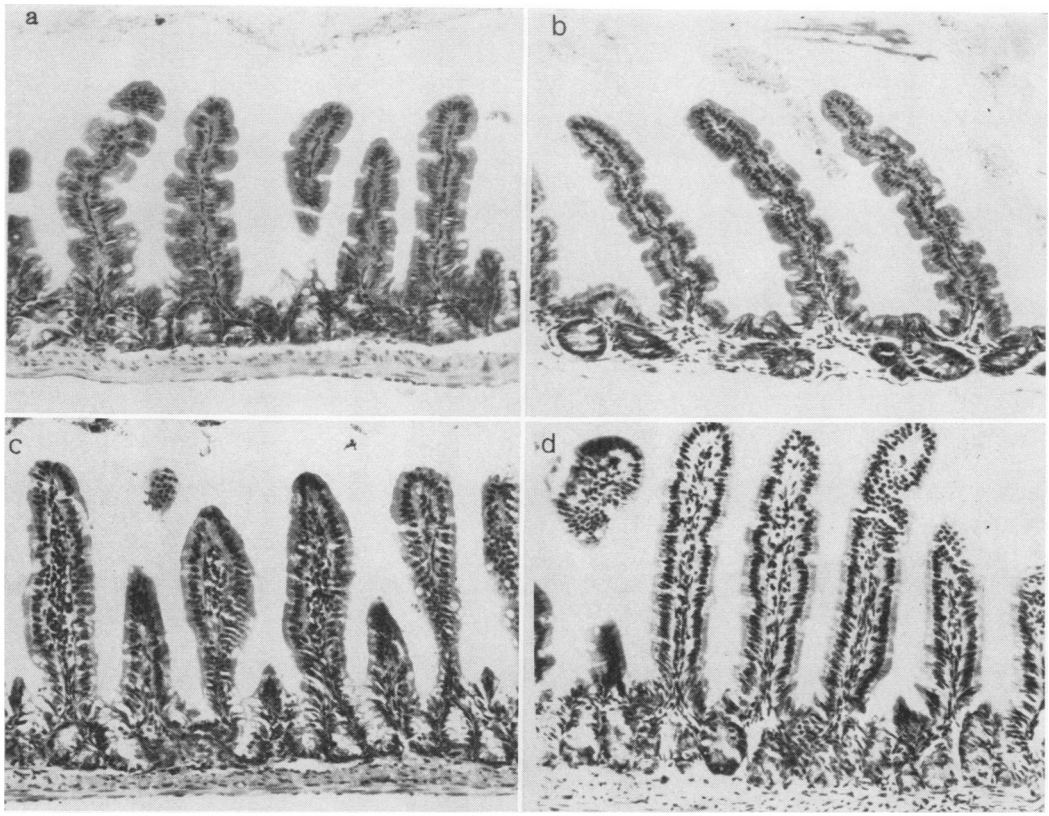

FIG. 2a. *Section of ileal mucosa of a germ-free mouse (group 1). The lamina propria of the villi is poorly developed, and the crypts of Lieberkuhn are shallow. Contrast with 2c and 2d. Hematoxylin and eosin, × 215.*

FIG. 2b. *Section of ileal mucosa of a mouse harboring E. coli (group 2). The appearance is similar to that shown in 2a. Hematoxylin and eosin, × 215.*

FIG. 2c. *Section of ileal mucosa of a mouse harboring a conventional flora (group 3). The lamina propria, with its "usual" cellularity, contrasts with that shown in 2a and 2b. The crypts are also deeper. Hematoxylin and eosin, × 215.*

FIG. 2d. *Section of ileal mucosa of a mouse harboring a defined flora (group 4). The histological appearance resembles that of the conventional mucosa illustrated in 2c. Hematoxylin and eosin, × 215.*

which are responsible for the "normal" features of the conventional mouse, at least within the limits of the criteria examined. It is unlikely that all of the bacteria used in the experiment shown in Table 5 are necessary for this effect. In fact, preliminary experiments have shown that this number may be reduced drastically. However, some strict anaerobes appear to be required because the facultative aerobes used (Table 5) were ineffective when established alone in the absence of strict anaerobes. It has been demonstrated (Table 1) that the establishment of at least some strictly anaerobic bacteria requires the presence of other bacteria. For this reason alone it seems likely that more than one species of microorganism is necessary to establish "normal" features in a germ-free mouse.

The requirement of a previously established *E. coli* flora for the successful implantation of a strictly anaerobic bacterium recalls the findings of Gibbons et al. (6), who showed that some anaerobic bacteria may be established in germ-free mice only as polycontaminants, not as monocontaminants. The simplest explanation for this finding would be that the *E. coli* strain used in the present experiments reduced the oxidation-reduction (O-R) potential of the intestinal contents to a level permitting growth of strict anaerobes. It is well known that the O-R potential in the cecum of germ-free mice is relatively high (7) and that *E. coli* is very active in reducing its environment (5).

One may conclude from the above studies that the population of *E. coli* in the gastrointestinal tract of the mouse is a function of the multiplication of this bacterium in the cecum, and perhaps the lower ileum, and that the sizable *E. coli* population in the stomach is largely depend-

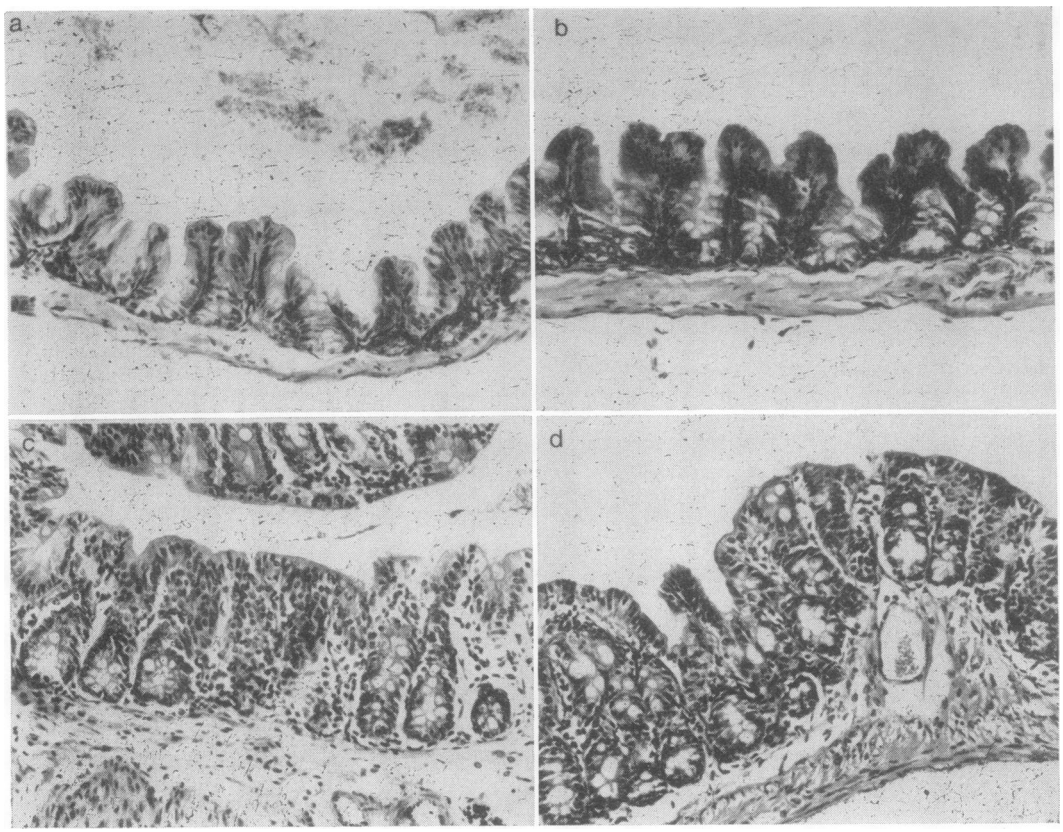

FIG. 3a. *Section of cecal wall of a germ-free mouse (group 1). The entire wall is thin, the mucosa somewhat villous, and the lamina propria poorly developed. Contrast with 3c and 3d. Hematoxylin and eosin, × 215.*

FIG. 3b. *Section of cecal wall of a mouse harboring E. coli (group 2). The appearance is similar to that shown in 2a. Hematoxylin and eosin, × 215.*

FIG. 3c. *Section of cecal wall of a mouse harboring a conventional flora (group 3). The thicker wall, the mucosal architecture, and the cellularity of the lamina propria contrast with the appearance of 3a and 3b. Hematoxylin and eosin, × 215.*

FIG. 3d. *Section of cecal wall of a mouse harboring a defined flora (group 4). The histological appearance resembles that of the conventional mucosa illustrated in 3c. Hematoxylin and eosin, × 215.*

ent on that of the cecum. The cecal population, in turn, appears to be controlled by the other bacteria normally present at this site. The data presented indicate that these "other" bacteria must include anaerobes in order to be effective. The data presented in Tables 4–6 allow some preliminary conclusions as to the mechanism by which the normal bacteria control the *E. coli* population. Meynell (8) concluded from studies on the growth rate of *Salmonella* in the normal and streptomycin-treated mouse intestine that the mechanism controlling the intestinal population of enterobacteria was based on the inhibition of these microorganisms by volatile fatty acids. The present findings are at variance with this hypothesis. Regardless of the type of flora established, the population of *E. coli* C25 became stable at a density which was usually lower than that in mice monocontaminated with *E. coli* C25. If the cecum is looked upon as a mixed continuous-flow culture, as was done by Meynell, a stable *E. coli* population would, of course, imply that the growth rate of *E. coli* was unimpaired even when its population size was much reduced. In fact, since normal mice have been found to show a faster intestinal transit time than germ-free animals (1) the growth rate of *E. coli* in mice contaminated with other bacteria may actually have been somewhat higher than that in monocontaminated mice (even though the population density was much higher in the latter case). This finding is, of course, incompatible with the presence of a fatty acid inhibitor which functions to reduce the overall rate of bacterial multiplication in a completely mixed system. It is, however, compatible with the assumption that the

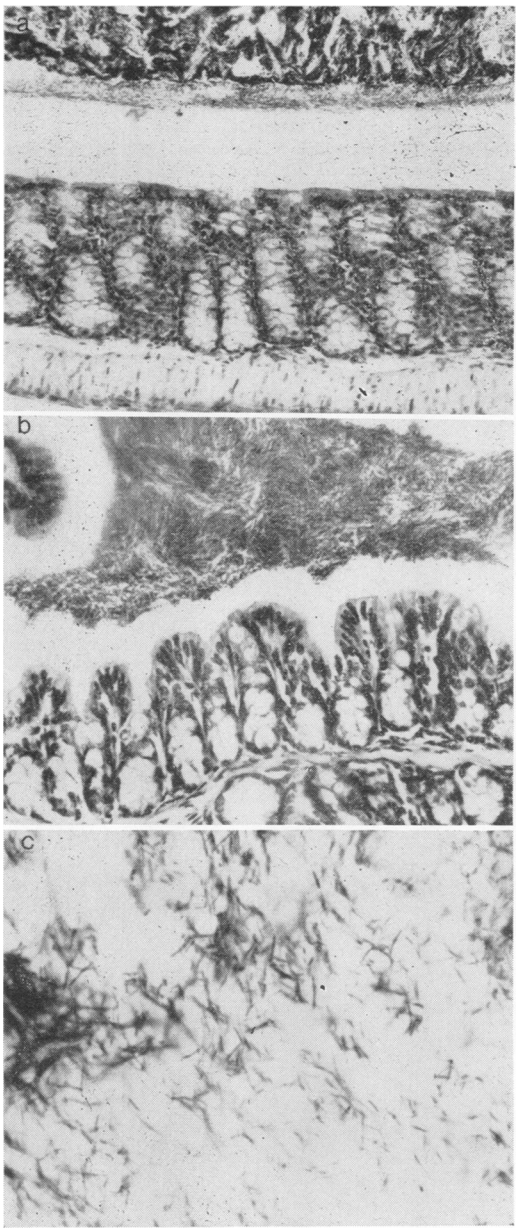

FIG. 4a. *Section of colon of a mouse harboring a conventional flora (group 3). The blank space across the mid-portion of the picture represents a shrinkage artifact. The light granular layer immediately above this space represents a feltwork of bacteria. The uppermost darker layer is fecal. Hematoxylin and eosin, × 190.*

FIG. 4b. *Section of colon of a mouse harboring a defined flora (group 4). The dense mass above the shrinkage space is a feltwork of bacteria comparable to that shown in 4a. Hematoxylin and eosin, × 190.*

FIG. 4c. *Serial section of the bacterial layer shown in 4b. The bacterial population consists of long, slender, or slightly tapered rods. Methylene blue, × 915.*

size of the *E. coli* population is controlled by a limiting nutrient, a mechanism which has been found to function in *E. coli-Shigella* interactions in the mouse intestine (5).

An alternate explanation for the above findings may be reached, if one assumes that the cecum is not analogous to a perfectly mixed continuous-flow culture. Most likely, bacteria associated with the mucosa would be washed out at a lower rate than those in the lumen and, consequently, could maintain a stable population at a lower growth rate than those multiplying in the lumen. Thus, reduction of the overall growth rate by fatty acids or some other inhibitory mechanism may eliminate the population of a given bacterial species from the lumen but still allow a constant number of bacteria to be shed from the mucosa-associated part of the original population. If this occurred in vivo, it would, of course, invalidate any conclusions drawn from the simple mathematical model used by Meynell (8). However, in the case of *E. coli*, such a situation appears less likely because, as discussed above, the *E. coli* used in the present studies did not form an extensive mucosa-associated flora comparable to that found with certain other bacteria.

It is obvious from this discussion that the question of control mechanisms operating in the intestinal flora requires intensive future study. The data presented in the present paper provide the necessary tools for such studies by showing that the bacteria involved in the control process can be isolated in pure culture and can be reimplanted into germ-free mice to form a known flora with "normal" properties.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants AI-07328 and AI-07631 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Abrams, G. D., and J. E. Bishop. 1967. Effect of the normal microbial flora on gastrointestinal motility. Proc. Soc. Exp. Biol. Med. 126:301–304.
2. 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. Microbiol. 17:568–576.
3. Donaldson, R. M. 1964. Normal bacterial populations of the intestine and their relation to intestinal function. N. Engl. J. Med. 270:938 and 994.
4. Dubos, R., R. W. Schaedler, R. Costello, and P. Hoet. 1965. Indigenous, normal and autochthonous flora of the gastrointestinal tract. J. Exp. Med. 122:67–75.
5. Freter, R. 1962. In vivo and in vitro antagonism of intestinal bacteria against Shigella flexneri. J. Infec. Dis. 110:38–46.
6. Gibbons, R. J., S. S. Socransky, and B. Kapsimalis. 1964. Establishment of human indigenous bacteria in germ-free mice. J. Bacteriol. 88:1316–1323.
7. Gordon, H. A. 1968. Is the germ-free animal normal? A review of its anomalies in young and old age, p. 127–150.

In M. E. Coates (ed.), The germ-free animal in research. Academic Press Inc., New York.

8. Meynell, G. G. 1963. Antibacterial mechanisms of the mouse gut. II. The role of EH and volatile fatty acids in the normal gut. Brit. J. Exp. Pathol. 44:209–219.
9. 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. Infec. Dis. 113:59–66.
10. Moore, W. E. C. 1969. Current research on the anaerobic flora of the gastrointestinal tract. *In* The use of drugs in animal feeds. Publication 1679, National Academy of Sciences, Washington, D.C.
11. Plaut, A. G., S. L. Gorbach, L. Nahas, and L. Weinstein. 1967. Studies of intestinal microflora. Gastroenterology 53:868–873.
12. Prevot, A., and V. Fredette. 1965. Manual for the classification and determination of the anaerobic bacteria. Lea and Febiger, Philadelphia.
13. Savage, D. C., R. Dubos, and R. W. Schaedler. 1968. The gastrointestinal epithelium and its autochthonous bacterial flora. J. Exp. Med. 127:67–76.
14. Smith, L. D. S., and L. V. Holdeman. 1968. The pathogenic anaerobic bacteria. Charles C Thomas, Publisher, Springfield, Ill.
15. Spears, R. W., and R. Freter. 1967. Improved isolation of anaerobic bacteria from the mouse cecum by maintaining continuous strict anaerobiosis. Proc. Soc. Exp. Biol. Med. 124:903–909.
16. Virginia Polytechnic Institute Laboratory. 1969. Outline of clinical methods in anaerobic bacteriology. The Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg.