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Microbial Colonization of the Intestinal Epithelium in Suckling Mice

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Colonization by indigenous microorganisms of the mucosal epithelia of the large bowels of suckling mice was followed by microbial culture techniques and by light, fluorescence, and electron microscopy. Certain microbes colonize in distinctive patterns the cecal and colonic epithelia in these mice. Coliforms and enterococci colonize the large bowel 7 to 9 days after birth and reach high population levels during the second week. During that period, these facultative anaerobes can be detected by immunofluorescence techniques in microcolonies in the mucin on the epithelium. During the third week, however, after their populations decline to the low levels characteristic of adult mice, coliforms and enteroccoci can be observed only infrequently in the mucous layer. Anaerobic fusiform-shaped bacteria appear in the mucous layers along with the microcolonies of enterococci and coliforms during the second week after birth. These anaerobes increase in numbers in the mucin until they form thick layers on the mucosal epithelium by the end of the third week. They remain in the mucous layer throughout the life of the normal mouse. Anaerobic spiral-shaped microbes also colonize the mucous layer on the cecal and colonic epithelium. But these organisms can be detected by immunofluorescence in 1-week-old mice, well in advance of the time the fusiform-shaped bacteria can be found. In the second week, the latter microbes co-inhabit the mucous layer with the spiral-shaped organisms. The fusiform- and spiral-shaped microbes remain associated in the mucin on the cecal and colonic mucosal epithelia into the adult life of mice.

The gastrointestinal canals of suckling mice from certain specific pathogen-free colonies are colonized in a particular sequence by various types of indigenous bacteria. Lactobacilli and anaerobic streptococci colonize first in 1 or 2 days after birth (6, 9). These microbes are found throughout the tract, but particularly in the stomach, where they form a layer on the keratinized stratified squamous epithelium of the nonsecreting tissue (6). The lactic acid bacteria are followed by coliforms and enterococci a few days later (6, 9). The latter bacterial types are followed by oxygen-tolerant and oxygen-intolerant anaerobic bacteria during the second week after birth (3, 6). The oxygen-tolerant bacteria are predominantly bacteroides; the oxygen-intolerant forms are fusiform-shaped bacteria of several genera. One or more types of these fusiform-shaped bacteria colonize the mucin closely associated with the epithelium of the mucosa of the ceca and colons of adult mice (6, 7).

Anaerobic spiral-shaped microorganisms also can be detected in the ceca and colons of adult mice (3, 4, 7). Both oxygen-tolerant and oxygen-intolerant forms are present (7). In adult animals, at least one type of spiral-shaped microbe colonizes the mucous layer intermixed with the fusiform-shaped bacteria (7). In this paper, we report that in suckling mice one type of spiral-shaped microorganism colonizes the epithelial mucus during the first week after birth, and the fusiform-shaped bacteria colonize at least a week later. In addition, we report that coliforms and enterococci also can be found on the epithelial mucin of the ceca and colons of baby mice when the population levels of these facultative anaerobes are at high levels during the second week after birth.

MATERIALS AND METHODS

Mice. ARS Ha/ICR (A. R. Gibco, Madison, Wis.) specific pathogen-free (SPF) females were bred in our own colony. Pregnant SPF CF-1 (Carworth-Bioquest, New City, N.Y.) and CD-1 (Charles River, Wilmington, Mass.) females were purchased directly from the suppliers. Animals from the three colonies were kept separated from each other at all times in our laboratory. They were housed in plastic boxes with Isocaps (Isocage, Carworth) containing Ad-Sorb Dri (Allied Mills, Chicago, Ill.) in separate flexible film isolators (Standard Safety Equipment Co., Pala-

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tine, Ill.) previously sterilized with 2% peracetic acid. All entering and exiting air was filtered through glass wool. Lab Blox (Allied Mills) and acidified water (7) were given ad libitum. The date of birth of the infants was recorded so that experiments could be conducted with animals of precisely known age.

Preparations of bowel specimens. Animals were killed with either chloroform vapors or CO_2 and autopsied. Ceca and colons were removed and treated in one of three ways. For bacteriological culturing, the ceca, with contents intact, were weighed and then homogenized with Teflon grinders in 5 ml of sterile charcoal water (8). For immunofluorescence testing, the ceca and colons with contents intact were frozen at −22 C in 2% methyl cellulose in saline (6). For electron microscopy, pieces of ceca and colon from individual mice were homogenized together in 2 ml of cold phosphate-buffered 2% glutaraldehyde.

Bacteriological culture techniques. The homogenates described above were diluted in charcoal water in 10-fold steps. Calibrated loopfuls of each dilution were then spread onto Tergitol-7-triphenyl tetrazolium medium (T_7T) selective for coliform bacteria and methylene blue medium selective for enterococci (9). Inoculated plates of both media were incubated aerobically at 37 C for 24 h. Estimates of the bacterial populations contained in each gram of cecal tissue were made from counts of colonies appearing on the media (9).

Histological methods. Segments of colons or whole ceca were frozen with contents intact in 2% methyl cellulose in saline. The tissues were sectioned at 4 μm on a microtome cryostat and fixed onto slides in absolute methanol. These slides were stored overnight at 4 C. Slides were then processed for either immunofluorescence testing or tissue Gram staining.

Reagents for immunofluorescence. Rabbit antiserum containing antibodies to a spiral-shaped bacterium isolated from Ha/ICR mice (7) was used in an indirect immunofluorescence test for spiral-shaped microbes. The preparation of this antibody and its absorption with fusiform-shaped bacteria and mouse liver powder have been described (7). Normal rabbit serum and a preparation of goat anti-rabbit IgG labeled with fluorescein isothiocyanate (FITC; FITC-labeled goat anti-rabbit IgG, kindly supplied by W. J. Mandy) were used in the indirect test. The labeled antiserum was also absorbed as described above.

FITC-labeled immunoglobulin preparations prepared from the sera of rabbits immunized with coliforms or enterococci were used for direct immunofluorescence testing for these bacterial types. The coliform and enterococcal vaccines were prepared from an *Escherichia coli* strain and a *Streptococcus* species isolated from Ha/ICR mice. The bacterial cells of both types were grown in quantity in penicillin assay broth (Difco), centrifuged, and washed three times with phosphate-buffered saline (PBS). The coliform cells in PBS were heat-killed at 60 C for 30 min and then adjusted to approximately 2×10^{10} cells/ml. The enterococcal cells were killed with 1.2% formaldehyde in PBS, washed three times with PBS, and stored in 0.1% formaldehyde in PBS. This enterococcal vaccine was adjusted to approximately 4×10^9 cells/ml.

Sera containing antibodies to the *E. coli* strain were obtained from 2.5-kg rabbits given three intravenous injections of 1×10^{10} cells of the coliform vaccine. The rabbits were rested for 4 days between each injection and bled 5 days after the last injection. Sera from individual rabbits were pooled. Pooled sera used to prepare the immunofluorescence reagent agglutinated the coliform in suspension to a titer of 1:512.

Sera containing antibodies to the enterococcal *Streptococcus* strain were obtained from 2.5-kg rabbits given 12 intravenous injections ranging from 1×10^9 to 4×10^9 cells of the enterococcal vaccine. The rabbits were injected on each of three successive days and then rested for 9 days. This sequence was repeated four times for each animal; the rabbits were bled 6 days after the last injection. Individual sera were pooled. Pooled sera used to prepare the immunofluorescence reagent agglutinated the enterococcal cells to a titer of 1:2,048.

Immunofluorescence reagents were prepared by the same procedure from the anticoliform, antistreptococcal, and normal rabbit sera. Globulins were precipitated from the sera with ammonium sulfate, conjugated with FITC, and dialyzed (1). The conjugated globulins were diluted 1:1 with PBS for use in the direct fluorescent-antibody stain. Both reagents specifically stained their homologous antigen. They did not stain other indigenous microorganisms from Ha/ICR mice including *Lactobacillus* species, slow lactose-fermenting *E. coli* and *Bacteroides* species. Both reagents stained slightly the animal tissue in histological sections of the bowels. This small amount of nonspecific fluorescence did not interfere in the process of localizing the bacteria on the mucosal epithelium.

Immunofluorescence testing. Ceca or colons were frozen and sectioned (6). For indirect testing for spiral-shaped microbes, the sections were flooded with either normal rabbit serum or serum containing antibodies to the spiral-shaped bacterium, and then incubated for 30 min at 37 C in high humidity (1). The sections were then washed well with PBS and flooded with FITC goat anti-rabbit IgG. The slides were again incubated for 30 min at 37 C and again washed well with PBS. For direct testing for coliforms or enterococci, the sections were flooded with one of the immunofluoresence reagents prepared as described above, incubated at 37 C, and then washed well with PBS. Cover slips were mounted onto all the slides with PBS glycerine. All sections were examined with a Leitz Ortholux fluorescence microscope fitted with a mercury vapor light source, 3-mm BC 12 excitor filter and a K460 barrier filter (E. Leitz, New York, N.Y.).

Electron microscopy. A drop of homogenized

cecum and colon in phosphate-buffered 2% glutaraldehyde was placed onto Formvar-coated grids. After a few minutes, the excess solution was gently removed with filter paper, and a drop of 0.1 to 1% phosphotungstic acid was placed on the grid. The phosphotungstic acid was removed within 30 s with filter paper. The grids were viewed in a Hitachi HU-8 electron microscope.

RESULTS

Colonization of the cecal epithelium by coliforms and enterococci. Enterococci and coliforms can be cultured from ceca or colons of baby Ha/ICR mice beginning about 9 days after birth (Table 1). The populations of both bacterial types usually increase to levels of 10^6 to 10^7 bacteria per g of whole bowel by the 11th or 12th day after birth, and remain at those levels until the 19th to the 21st day. Thereafter, they drop to levels of 10^5 to $< 10^3$ organisms per g, characteristic of adult mice (6).

Immunofluorescence detection of the coliforms and enterococci in the mucus on the cecal or colonic epithelium of the baby mice parallels the culture results (Table 1). On about the 10th day after birth, a few of these bacteria can be seen in the mucous layer and also in the lumen. Thereafter, for 7 or 8 days, microcolonies of each type could be seen with ease in the lumen and in the mucus adjacent to the epithelial cells of the mucosa. Microcolonies consist of several closely associated fluorescing cells. When the bacterial population levels dropped to adult levels in mice 19 to 21 days old, the enterococci and coliforms could be observed only infrequently in the lumen and in the mucus on the epithelium. At this time, and in 30-day-old animals, they could be seen occasionally as single cells mixed in with massive numbers of fusiform- and spiral-shaped bacteria.

Colonization of cecal and colonic epithelia by fusiform-shaped bacteria. Fusiform-shaped bacteria are known to colonize the mucous layer on the epithelium of the murine large bowel in the second week after birth. These microbes pack the mucous layer in massive numbers; their populations quickly reach high levels in the bowel and remain at those levels throughout the life of the animal (5, 6). Using tissue Gram stains in this study, we first detected these bacteria, mixed in with coliforms and enterococci in the mucous layer, at the end of the first or beginning of the second week after birth (Table 2). By the 11th day, these fusiform-shaped microbes are easily detectable in most of the animals (Fig. 1).

Colonization of cecal and colonic epithelia by spiral-shaped microorganisms. Spiral-shaped organisms can be observed by immunofluorescence as early as 3 days after birth in CD-1 and CF-1 mice (Table 3). They appear as early as 5 days after birth in Ha/ICR mice. In 3- to 7-day-old animals, they can be seen occasionally as single cells or in small microcolonies in the mucus on the cecal or colonic epithelium. During the next week, however, they can be found with ease in the mucous layer. At no time can these microbes be seen with ease in the lumen of the cecum or colon.

Until the 10th or 11th day after birth, these spiral-shaped organisms were virtually alone in the mucous layer. By the 11th day, however, some coliforms, enterococci, and fusiform-shaped bacteria occasionally could be seen along with the spiral-shaped microbes in the mucin in some mice. By about 2 weeks after birth, almost every animal examined showed fusiform- and spiral-shaped organisms in the mucous layer adjacent to the mucosal epithelial cells. This pattern was

TABLE 1. *Detection of coliforms and enterococci colonizing the large bowels of baby Ha/ICR mice*[a]

Bacterial type	Detection method	Days after birth									
		3	5	7	9	11	13	15	17	19	21
Coliforms	Immunofluorescence	0/3[b]	0/6	1/9	0/9	3/9	4/9	5/9	6/9	6/6	2/3
	Culture	ND	0/3	0/6	1/6	3/6	4/6	3/5	5/6	3/3	2/3
Enterococci	Immunofluorescence	ND	0/3	0/6	0/6	2/6	1/6	4/6	3/6	3/3	2/3
	Culture	ND	0/3	0/6	1/6	2/6	5/6	4/5	1/6	3/3	1/3

[a] Coliforms and enterococci were detected by two methods. (i) Frozen tissue sections of ceca or colons were treated with either FITC-anticoliform antibody or FITC-antienterococcal antibody. (ii) Homogenized pieces of colon or cecum and colon together were diluted in charcoal water (8) and spread onto plates of T_7T or methylene blue medium; population levels above 10^3 bacteria per g of whole bowel are detected by this method.

[b] Ratio represents the number of mice in which the bacterial type was detected/number of animals examined. ND, Not done.

observed as well in weanlings and adults of all three strains of mice examined.

Immunofluorescence was used to locate these small, spiral-shaped organisms because they cannot be seen readily in frozen histological sections (7). On rare occasions, however, they can be viewed with dark-field optics when the mucus in suckling mice is not yet densely populated with other microorganisms (Fig. 2).

TABLE 2. *Detection of fusiform-shaped bacteria colonizing the ceca and colons of Ha/ICR, CD-1, and CF-1 mice*[a]

Mouse strain	Area of bowel	Days after birth					
		3	5	7	9	11	30
Ha/ICR	Cecum	0/5[b]	0/5	1/5	1/5	4/5	ND
	Colon	0/5	0/5	0/5	0/5	4/5	ND
CD-1	Cecum	0/5	0/5	0/5	0/5	3/5	5/5
	Colon	0/5	0/5	1/5	0/5	1/5	5/5
CF-1	Cecum	0/5	0/5	0/5	0/5	4/5	5/5
	Colon	0/5	0/5	1/5	1/5	3/5	5/5

[a] Fusiform-shaped bacteria were detected in frozen histological sections of ceca and colons stained by a tissue Gram stain (6).

[b] Ratio represents the number of animals possessing fusiform-shaped bacteria/number of animals examined.

Detection of spiral-shaped microbes in cecal homogenates from suckling mice by electron microscopy. Ceca with their contents intact were removed from suckling mice, homogenized, and negatively stained with PTA. With an electron microscope, spiral-shaped microorganisms could be detected in these homogenates only 2 to 4 days before fusiform-shaped organisms could be observed (Table 4), in contrast to findings with the immunofluorescence technique. Thus, the technique is not as sensitive as immunofluorescence in detecting the microbes in the intestinal tract. The spiral-shaped microbes probably would be detectable in mice younger than 7 days if the total volume of the cecal homogenates were negatively stained. Far too many grids would have to be examined by electron microscopy, however, for this procedure to be practical.

The spiral-shaped microorganisms from all three strains of suckling mice are similar in their

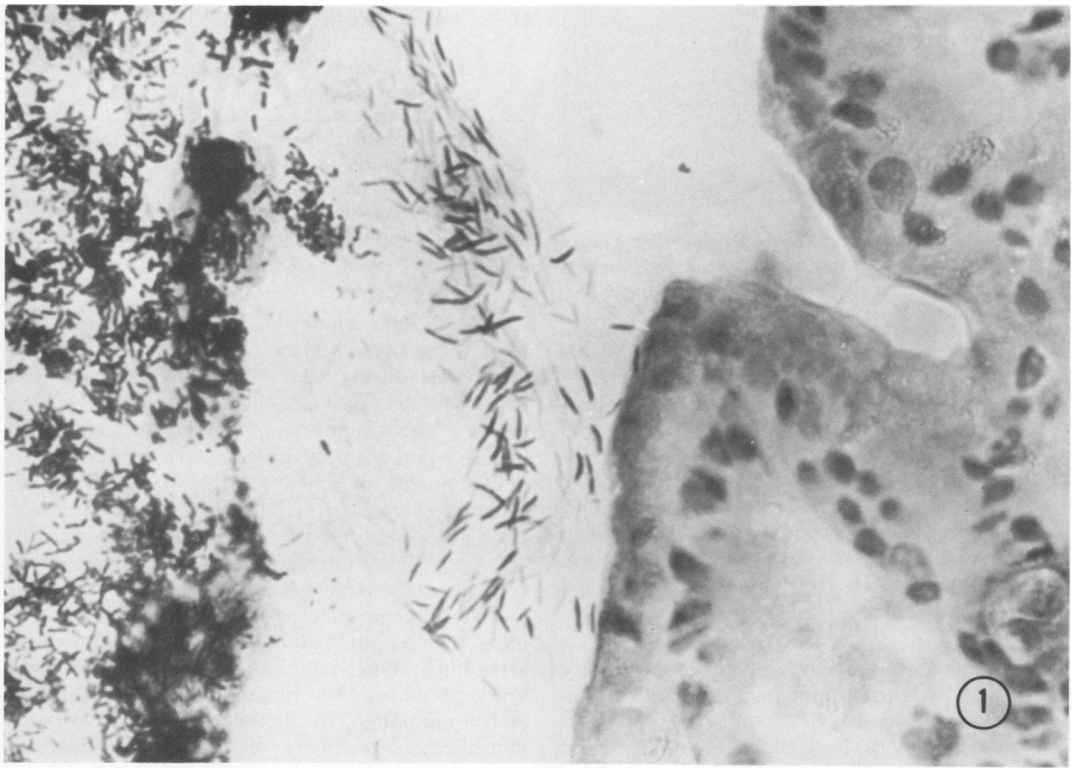

FIG. 1. *Fusiform-shaped bacteria, in the mucin between the mucosal epithelium to the right and the mass of microbes in the lumen to the left, in a frozen section of the colon of an 11-day-old CD-1 mouse.* × *2,200.*

TABLE 3. *Immunofluorescent detection of spiral-shaped microbes colonizing the ceca or colons of baby and adult mice*[a]

Mouse strain	Days after birth										
	3	5	7	9	11	13	15	17	21	30	50
Ha/ICR	0/8[b]	1/6	5/14	11/14	14/14	9/9	9/9	9/9	3/3	ND	5/5
CD-1	3/5	5/5	5/5	5/5	5/5	ND	ND	ND	ND	5/5	ND
CF-1	3/5	3/5	5/5	5/5	5/5	ND	ND	ND	ND	5/5	ND

[a] Spiral-shaped microbes were detected by indirect immunofluorescence in frozen sections of ceca or colons of individual mice (7). If the organisms were present in any section, the mouse was recorded as possessing spirals.

[b] Ratio indicates the number of mice possessing spiral-shaped microbes in their ceca or colons/number of ceca and colons examined.

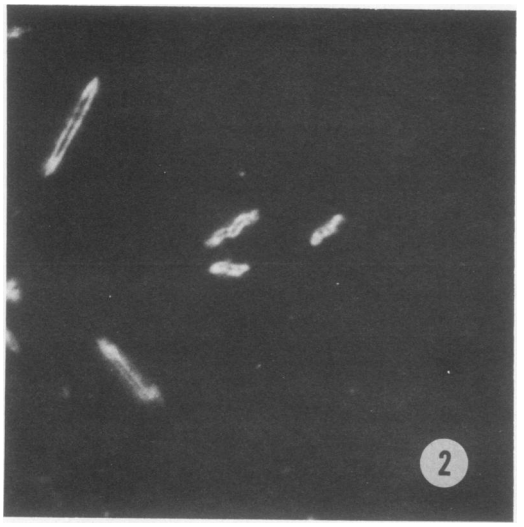

FIG. 2. *Fusiform- and spiral-shaped microbes as visualized by dark-field microscopy near the mucosal epithelium in a frozen section of the colon of a CD-1 suckling mouse.* ×*5,100.*

TABLE 4. *Detection of spiral and fusiform-shaped microbes in ceca of suckling mice by electron microscopy*[a]

Mouse strain	Morphological type of microbe	Days after birth			
		5	7	9	11
Ha/ICR	Spiral	0/5[b]	2/5	2/5	5/5
	Fusiform	0/5	0/5	0/5	3/5
CD-1	Spiral	0/5	3/5	5/5	5/5
	Fusiform	0/5	0/5	1/5	3/5
CF-1	Spiral	0/5	1/5	3/5	5/5
	Fusiform	0/5	0/5	1/5	5/5

[a] Spiral- and fusiform-shaped microbes were detected in negatively stained cecal homogenates examined in an electron microscope.

[b] Ratio represents the number of mice possessing the morphological type/number of mice examined.

morphology (Fig. 3a). They have polar flagella and usually have two to seven "S" curves along the cell. They range from approximately 2.0 to 7.0 μm in length and from 0.15 to 0.30 μm in diameter. Most of the organisms have one to five circular bodies inside the protoplasmic cylinder. A few of these bodies show variable electron densities, especially in those microbes seen in homogenates from Ha/ICR mice (Fig. 3b).

DISCUSSION

The colonization of the intestinal canals by enterococci, coliforms, and fusiform-shaped bacteria has been described for mice from several different colonies (2, 5, 6, 9). The coliforms and enterococci begin to colonize the large bowels of suckling mice around the end of the first week of life, reach high population levels from about the 10th to 17th days after birth, and then fall to low levels during the third week. During the times the populations of these facultative anaerobes are at high levels, microcolonies of gram-negative and gram-positive bacteria can be found in the mucin on the mucosal epithelium of the cecum and colon (6). Our findings from immunofluorescence studies confirm an earlier suspicion (6) that these microcolonies are composed of coliform and enterococcal bacteria.

Fusiform-shaped microorganisms begin to colonize the ceca and colons during the second week after birth. Their populations quickly reach high levels at which they remain throughout the life of the animal (5, 6). These microbes have been identified as members of the genera *Fusobacterium*, *Clostridium*, and *Eubacterium* (3). Some species of these anaerobic genera colonize the

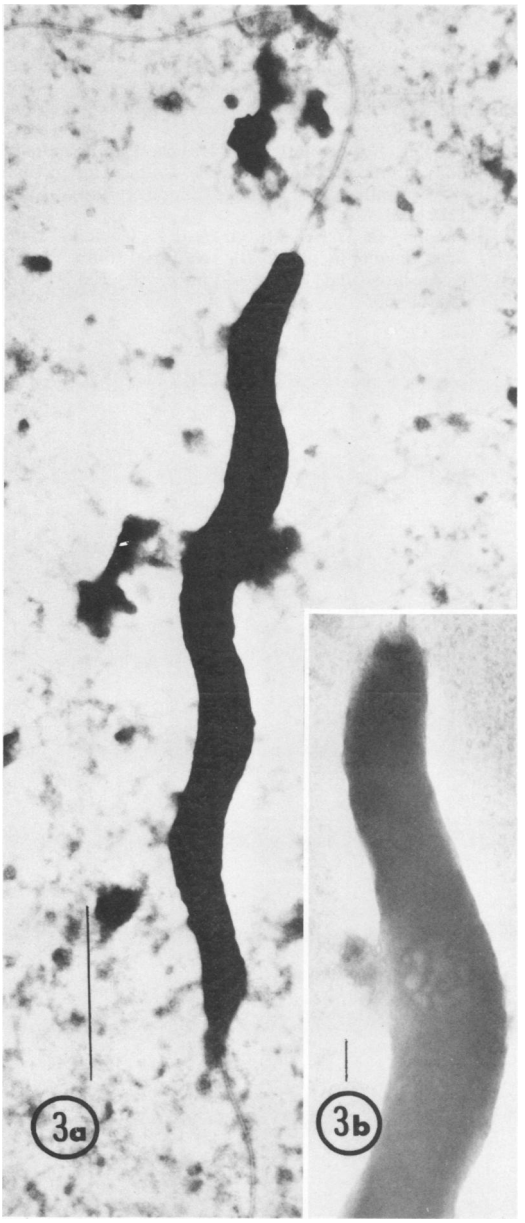

FIG. 3. *Spiral-shaped bacteria in negatively stained homogenates of cecal mucosa from Ha/ICR suckling mice. a, Bar represents 1 μm. ×23,000. b, Bar represents 0.1 μm. ×56,000.*

mucin on the mucosal epithelium of the cecum and colon. In doing so, apparently they displace the coliforms and enterococci from the mucin in the suckling mice.

Spiral-shaped microbes have been detected repeatedly in the large bowels of mice (3–5, 7). Such organisms have been shown to colonize the mucous layer in the large bowels (7). In homogenates from suckling CD-1 mice viewed by phase-contrast microscopy, the spiral-shaped bacteria could be observed in the bowels of mice as early as 12 days after birth (5). The specific immunofluorescence technique is a more sensitive method of detection, however, and allows us not only to discern the location of the spiral-shaped bacteria in the large bowel but also to detect the spirals when they are present in low numbers and further obscured by mucus (7). Using this technique, we find that the spiral-shaped organisms appear during the first week after birth and localize in the mucous layer adjacent to the mucosal epithelium about 2 to 5 days before the epithelium is colonized by fusiform-shaped organisms. Electron microscopy of negatively stained cecal homogenates showed a similar colonization pattern, except that the spiral-shaped microbes could not be detected before the end of the first week after birth. The microbes could not be detected earlier, in all probability because they were present at population levels too low to be detected by electron microscopy.

The negative stains indicated that the morphology was similar in the spiral-shaped organisms from the three different types of mice. The organisms were approximately 2 to 7 μm long with two to seven spirals, and they usually had two polar flagella. Many also had compartmented circular bodies within the protoplasmic cylinder.

The spiral-shaped microbe used to make the vaccine employed in this study was anaerobic but not oxygen-intolerant (7). Although the spiral-shaped microbes have yet to be cultured from suckling mice, they also may be anaerobic but not oxygen-intolerant. Oxygen tolerance may be a factor in the ability of these spiral-shaped microbes to colonize suckling mice early in life. All microbes, including lactobacilli, anaerobic streptococci, coliforms, enterococci, and these spiral-shaped bacteria, that colonize the murine gastrointestinal canal during the first week after birth, may be involved in developing an environment in the large bowel that favors the later establishment of the oxygen-intolerant fusiform-shaped anaerobes. The microaerophilic, facultatively anaerobic, and oxygen-tolerant anaerobic bacteria may contribute to the environment by removing oxygen, lowering the oxidation-reduction potential, and providing certain nutrients essential to the oxygen-intolerant anaerobes. The spiral-shaped microbes may be especially important in the development of this ecosystem.

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