Timing, Localization, and Persistence of Colonization by Segmented Filamentous Bacteria in the Neonatal Mouse Gut Depend on Immune Status of Mothers and Pups

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As a member of the indigenous gut mucosal microbiota, segmented filamentous bacteria (SFB) colonize the guts of a variety of vertebrates and invertebrates. They are potent microbial stimuli of the gut mucosal immune system. In the small intestines of mice and rats, it has been observed that SFB are absent during the suckling period and appear in high numbers shortly after weaning, then quickly retreat to the cecum and large intestine. In this study, we explored whether this microecological phenomenon resulted from the interaction between SFB and the passively acquired maternal mucosal immunity and/or the actively acquired mucosal immunity. We set up a mouse model by reciprocal crossings and backcrossings of SFB-monoassociated, formerly germ-free, immunocompetent (+/+ BALC/c mice and immunodeficient (scid/scid) mice to produce pups which are either immunocompetent (scid/+ or immunodeficient (scid/scid) and are born either to immunocompetent (scid/+ mothers or to immunodeficient (scid/scid) mothers. We monitored the number of SFB on the mucosa of the small intestine in the four different groups of mice after birth, as well as the level of passively acquired antibodies, the active gut mucosal immune responses, and immunoglobulin A (IgA) coating of SFB in the gut. The results showed that, irrespective of whether the pups were scid/scid or scid/+ , SFB could be found earlier on the mucosa of the small intestine in pups born to scid/scid mothers, appearing from day 13 and rapidly reaching a climax around weaning time on day 28, compared to the significantly delayed colonization in the pups of scid/+ mothers, starting from day 16 and peaking around days 28 to 32. After the climax, SFB quickly declined to very low levels in the small intestines of scid/+ pups of either scid/scid mothers or scid/+ mothers, whereas they remained at high levels in scid/scid pups at least until day 70, the last observation time in this study. The dynamic changes in SFB colonization of the small intestines of the different groups of pups may be related to the dynamic changes in the levels of SFB coated with secretory IgA (sIgA), which resulted from the significantly different levels of sIgA obtained from the mothers’ milk during the suckling period and, later, of self-produced sIgA in the small intestine. Nevertheless, it is evident that the timing, localization, and persistence of colonization of the neonatal gut by SFB depends on the immune status of both mothers and pups.

The intestinal tracts of animals and humans are heavily colonized by gut microbiota. Gut-associated lymphoid tissue (GALT), which generates strong mucosal immune responses against the invasion of frank pathogens, seems to have evolved to permit the survival and propagation of the indigenous microbial populations in the gut (29). On the other hand, GALT in conventional animals is in a physiologically activated state (4, 36), in sharp contrast to the quiescent state of GALT in germ-free (GF) animals. Little is known about the mechanisms of the interactions between indigenous microbiota and the immune system. GF animals are invaluable tools with which to approach this issue (3, 29).

As a member of the indigenous gut mucosal microbiota, segmented filamentous bacteria (SFB) colonize a variety of vertebrates and invertebrates (5, 14, 22). SFB are gram-positive spore-forming obligate anaerobes. Because they have not been successfully cultured in vitro, their presence in the intestine cannot yet be quantified by common microbiological techniques and they have not been classified by the usual procedures. Based on 16S rRNA analysis, SFB have been identified as closely related to Clostridium species (32, 33). In mice and rats, SFB are firmly attached to the epithelial cells of the distal ileal mucosa by a specialized terminal “holdfast” segment but do not penetrate the brush border of the epithelial cell to cause any pathogenic effect (7, 13, 28). They also show a preference for attachment to the epithelium covering the lymphoid tissue of Peyer’s patches (PP) (9, 13). This intimacy suggests a possible relationship between SFB and the gut mucosal immune system (14). Our recent studies (35) showed that SFB monoassociated with GF mice initiated a prompt rise in germinal center reactions (GCRs) of PP, which were prolonged during the entire observation period, gradually waning until 188 days after monoassociation. A large amount of immunoglobulin A (IgA) was produced in PP and intestinal fragment cultures to levels 24 to 63% of those in, conventionally reared mice, but less than 1.4% of the total IgA could be shown to be SFB-specific IgA. CD4 T cells in PP are gradually activated to the level found in conventionally reared mice. These results confirm the previous findings that GF mice, monoassociated with SFB, had increased numbers of IgA-secreting cells in the intestine, had elevated IgA titers in the serum and intestinal secretions, and exhibited increased proliferation of mesenteric lymph node (MLN) cells (15). Furthermore, it has been demonstrated that SFB monoassociation with GF mice resulted in...
the activation of natural killer cells and an increase in Thy 1+, α/β T-cell receptor (TCR), CD8α/β T cells in the intraepithelial leukocyte (IEL) compartment with high constitutive cytotoxic activity (21, 37, 38).

Another interesting observation is the succession of SFB in the guts of neonatal mice and rats. It has been reported that SFB were absent during the suckling period and appeared in high numbers shortly after weaning (6, 16, 28), then dramatically decreased in the small intestine (11). By inoculating 4-week-old GF mice with SFB, Snell et al. found a similar decline in SFB occurring in the small intestine within the following weeks, while SFB remained constantly present in the cecum and the number of IgA-secreting cells concomitantly increased in the lamina propria of the small intestine (34). We have also found that formerly GF severe combined immunodeficient (SCID) adult mice, monoassociated with SFB, do not clear their SFB. Their naturally colonized neonates also do not clear SFB from their small intestine up to the age of 1 year (unpublished data). All these observations indicate a correlation between immune response and SFB colonization of the gut. We postulated that maternal immunity passively transferred during the suckling period, and the later development of self gut mucosal immunity may contribute to the dynamic changes in the localization of SFB in the gut.

To support our hypothesis, we devised a mouse model by reciprocal crossings of SFB-riboassociated, formerly GF, immunocompetent BALB/c (+/+ +) mice and immunodeficient (scid/scid) mice to produce genetically identical immunocompetent F1 pups (scid/+). Then either female F1 mice (scid/) or immunodeficient (scid/scid) mothers or to immunodeficient (scid/scid) mothers. Thus the four groups of mice developed under conditions where the influence of maternal immunity was either present or absent and the self competence of mucosal immunity was either present or absent. Our results show that the passively acquired maternal secretory IgA (sIgA) in the milk and self-produced sIgA in the intestine may be related to the localization of SFB in the gut. Irrespective of whether maternal or neonatal, humoral mucosal immune responses, or both, provide the mechanism, it seems clear that the timing, localization, and persistence of colonization of the neonatal gut by SFB depend on the immune status of both mothers and pups.

MATERIALS AND METHODS

Mice. GF BALB/c mice, originally obtained from E. Balish of the University of Wisconsin Gnotobiological Laboratory (Madison) and GF C.B17 scid/scid mice, from R. Orcutt of Taconic Farms (Germantown, N.Y.) were used as breeders in this study. All the mice for this study were bred and maintained in one Trexler flexible film isolator within the gnotobiotic facility in the Department of Biology at the University of Pennsylvania (Philadelphia). First, 8- to 10-week-old GF BALB/c mice and GF C.B17 SCID mice were monoassociated with SFB by orally inoculation of each mouse with 0.2 ml of a phosphate-buffered saline (PBS) suspension containing SFB which was made from cecal contents of SFB- monoassociated mice. Then SFB- monoassociated BALB/c mice were mated with SFB- monoassociated SCID mice to generate F1 scid/+ litterers. F1 scid/+ litterers were naturally gut colonized with SFB. By further matings of 8-week-old SFB- monoassociated F1 scid/+ mice with SFB- monoassociated scid/scid mice, F2 litterers were generated. They were either scid/+ or scid/scid and were born either to F1, scid/+ mothers or to scid/scid mothers. Observations were made on days 13, 16, 20, 28, 32, 36, 44, 54, and 70 after the birth of the F2 mice. At each time point, three to five pups from each group were analyzed. Mice were weaned from their mothers on day 28 after birth. The immunological status of F2 mice was determined by flow cytometric analysis of CD4 and CD19 expression.

Quantification of SFB in the small intestine. SFB cannot be enumerated by culture plate counting, since no in vitro cultivation method is available. We have used and modified an accepted microscopic assay developed by the Nijmegen group (12, 34). Briefly, the small intestine was removed and cut into two equal parts. In the distal part, three pieces of small intestine, each 1 cm long, were cut off from the beginning, middle, and end, respectively. Another two pieces of intestinal length of the same size were taken from the middle of the cecum and large intestine, respectively. The pieces were longitudinally cut, the intestinal contents were removed carefully with a pair of tweezers, and the intestinal mucosa was vigorously rubbed onto an area of 1.5 mm in diameter on a microscope slide. Then the smear was fixed by heat and Gram stained. SFB were counted on 50 fields for each smear under the microscope with a 100× oil immersion lens. For presentation in this paper, we have pooled data from the counting of three pieces of each small intestine and calculated the average; then we have calculated the mean ± standard error of the mean for three to five pups in each group at each time point. Thus, each data point depicted is based on counting of 450 to 750 fields compiled from 9 to 15 smears.

GALT organ fragment cultures. The general method of the GALT organ fragments was developed in our laboratory, has been described previously (23). Briefly, PP were excised from the small intestine, and 1-cm-long segments from the duodenum, jejunum, and ileum of each mouse were excised, opened longitudinally, and washed five times with Ca2+-, Mg2+-free PBS containing 0.01% gentamicin (GIBCO, Grand Island, N.Y.) and 10 mM HEPES to remove debris. Segments were then rinsed twice to three times in Ca2+-, Mg2+-free PBS containing 0.05% EDTA, 0.01% gentamicin, and 10 mM HEPES to remove the mucin layer and to denude the villi of epithelium. Finally the tissue fragments were rinsed twice in complete RPMI 1640 containing 10% fetal bovine serum (PBS) to remove the EDTA. Tissues were cultured in a sterile 24-well plate (Costar, Cambridge, Mass.) in 1 ml of complete Kennett’s HY medium (GIBCO) containing 10% FBS, 1.0% l-glutamine, 0.01% gentamicin, and 1.0% antibiotic-antimycotic solution (100 U of penicillin, 100 mg of streptomycin, and 0.25 g of amphotericin B per ml) for 7 days in 90% O2 and 10% CO2 at 37°C. One intact PP or one, ~3-mm2 piece of a segment from the duodenum, jejunum, or ileum was cultured per well. Culture fluids were frozen prior to assay.

RIA. The radioimmunoassay (RIA) used in our laboratory has been described previously, (8, 23). To determine total IgA antibodies, plates were coated with a goat anti-mouse Fab fragment (Southern Biotechnology Associates, Birmingham, Ala.). SFB sonicate was used as the coating antigen to determine the level of anti-SFB IgA. The method for preparing the SFB sonicate has been described previously (35). The 125I-labeled goat anti-mouse IgA (Southern Biotechnology Associates) was used to develop all assays.

FACS analysis. PP cells were prepared by teasing the tissue apart in complete RPMI 1640. Single-cell suspensions were made by passage through a cell strainer. Cells were then washed in complete RPMI and stained with appropriate dilution of FITC- or PE-conjugated reagent in PBS-azide for 30 min on ice. After a PBS wash, they were analyzed on a fluorescence-activated cell sorter (FACS) model IV flow cytometer (Becton Dickinson, Sunnydale, Calif.). Fluorescein isothiocyanate (FLU)-labeled peanut agglutinin (PNA) in conjunction with a phycoerythrin (PE)-conjugated anti-α chain was used to stain for germinal center B cells. The same PE-conjugated anti-α and FLU-labeled goat anti-mouse IgA (Southern Biotechnology Associates) were used to stain surface IgA-positive B cells. PE-conjugated anti-CD4 (Pharmlingen, San Diego, Calif.) and FLU-labeled anti-CD45RB (Pharmlingen) were used to stain surface IgA-positive B cells. Preparatory of supernatant from stomach contents. To determine IgA levels present in the milk which was suckled by the pups, each pup’s stomach was dissected and placed in 1.0 ml of PBS. Then it was cut open and strongly vortexed. The suspension of stomach contents was spun at 4°C, at 28,000 rpm for 10 min. The supernatant was then frozen before RIA for detection of natural IgA and IgA-specific FACS.

FACS for IgA coating of SFB. The intestinal contents collected from the small intestine, cecum, and large intestine were dissolved in PBS by vortexing. The bacterial suspension was centrifuged at 40 × g for 5 min. The supernatant was then washed twice with PBS. The bacterial suspension was prepared so as to have an optical density at 550 nm (OD550) of 1.0. Two hundred micro liters of the bacterial suspension was centrifuged and the bacteria were stained with FLU-conjugated goat anti-mouse IgA for 30 min on ice and then washed with PBS. Before analysis, propidium iodide was added to the samples to achieve a final concentration of 40 μg/ml. FACS analyses were conducted using a FACScan.
gating on the propidium iodide-positive events. The percentages of IgA-positive events were calculated.

**Statistical analysis.** Differences in SFB counts and in total IgA production in the small intestines of different groups of pups were calculated by Student’s *t* test.

**RESULTS**

**Dynamic changes in SFB localization on intestinal mucosa of scid/+ or scid/scid pups which were born either to scid/scid mothers or scid/+ mothers.** Figure 1 shows that, irrespective of whether the pups were scid/scid or scid/+, in the pups born to scid/scid mothers, SFB could be found on the mucosa of the small intestine starting on, day 13 and rapidly reached a climax around weaning time, day 28. No statistical differences were found between these two groups of pups up to day 28. Afterwards, the number of SFB in the small intestine quickly declined in scid/+ pups of scid/scid mothers, whereas SFB remained at high levels in scid/scid mothers until day 70, the last observation time point in this study. A significant difference in the number of SFB between these two groups was observed at late time points from day 32 on (P < 0.05). In both groups of pups born to scid/+ mothers, SFB colonization was delayed compared to that in the pups of scid/scid mothers. SFB appeared on day 16. After that, SFB were found abundantly in the cecum and large intestine in all groups of mice, showing stable levels during the whole study period (data not shown).

**Levels of natural IgA and SFB-specific IgA in stomach contents of pups.** Antibodies in the stomach contents of suckling pups closely reflected the maternal antibodies in the milk (17, 18). As shown in Fig. 2, natural IgA and SFB-specific IgA were not detectable in the stomach contents of pups born to scid/scid mothers, whereas in those of pups born to scid/+ mothers, both natural IgA and SFB-specific IgA were easily detected up to day 24.

**Development of GCRs and subsets of PP cells in scid/+ pups born either to scid/scid mothers or to scid/+ mothers.** To explore the perturbations of GALT following natural SFB colonization of the guts of immunocompetent scid/+ pups, the levels of GCRs and activation of CD4 T cells in PP were determined by using flow cytometry. We used PNA binding by B cells as a germinal center marker (31). We also checked the number and percentage of surface IgA+ B cells and activated CD4 T cells, which are represented by the CD45RBlow phenotype. The results shown in Fig. 3 demonstrate a prompt rise in SFB rapidly declined in the small intestine to a very low level in scid/+ pups of scid/+ mothers but remained at a significantly high level in scid/scid pups of scid/+ mothers until day 70.

In the cecum and large intestine, SFB could be found from day 13 on in pups born to scid/scid mothers. In pups born to scid/+ mothers, SFB appeared on day 16. After that, SFB were found abundantly in the cecum and large intestine in all groups of mice, showing stable levels during the whole study period (data not shown).

![FIG. 1. Numbers of SFB on the mucosae of small intestines from scid/scid pups (P−/−) and scid/+ pups (P+/-) born either to scid/scid mothers (M−/−) or scid/+ mothers (M+/−) under SFB-monoassociated conditions. Data are means ± standard errors of the means and represent total numbers of SFB counted from 50 fields on each smear of three 1-cm-long pieces of small intestine, which were taken, respectively, from the beginning, middle, and last part of the half distal small intestine. Three to five mice from each group were used for each time point.](http://iai.asm.org/)

![Diagram](http://iai.asm.org/)
in the percentage of germinal center B cells, surface IgA^+ B cells, and activated CD4^+ T cells in PP of scid/+ pups of scid/scid mothers, starting from day 16 and peaking on day 32, whereas in scid/+ pups of scid/+ mothers, the rise was delayed, starting from day 20 and peaking on day 36. Thereafter the percentages of germinal center B cells, surface IgA^+ B cells, and activated CD4^+ T cells in both groups gradually declined but stayed at similar levels during the observation period.

**Production of natural IgA and SFB-specific IgA in GALT of scid/+ pups born to scid/scid or scid/+ mothers.** We wanted to know whether the GCRs in PP stimulated by SFB resulted in a corresponding production of sIgA in the gut. We did fragment cultures of PP and segments of small intestine from scid/+ mice and detected the levels of natural IgA and SFB-specific IgA production in each culture. Figure 4A shows an early rise in natural IgA production by PP and the small intestine in scid/+ pups of scid/scid mothers. In pups of scid/scid mothers, a considerable amount of natural IgA, which was about 270 ng/ml in the culture of each small intestine segment, could be detected as early as day 20. This level rapidly increased to about 820 ng/ml on day 36 and remained at 500 to 600 ng/ml thereafter. In pups of scid/+ mothers (Fig. 4B), a later rise in natural IgA was observed from day 24, at which point the level was only about 65 ng/ml in the culture of a typical small intestine segment. Then it gradually reached the highest level of 900 ng/ml on day 44 and remained at 630 to 790 ng/ml thereafter. A significant difference in natural IgA production in the small intestines of these two groups of pups was observed from day 13 to day 24 (P < 0.01).

In scid/+ pups of both scid/scid and scid/+ mothers, production of SFB-specific IgA was extremely low but was detectable at days 24 to 28 (0.5 to 2.5 ng/ml). No significant difference was seen in the time course of SFB-specific IgA production between the two groups (data not shown).
SFB coating with sIgA in the small intestine. To determine whether the sIgA passively acquired from milk and the sIgA self-produced in the small intestine react to SFB in the guts of mice, we performed a FACS analysis for IgA coating of SFB. As shown in Fig. 5, scid/scid pups of scid/scid mothers contained SFB with no IgA coating. In scid/scid pups of scid/+ mothers, the level of SFB coated with maternal IgA rapidly increased before weaning and attained the highest level around day 24. It then declined around day 28 and eventually went down to a negligible level like that in scid/scid pups of scid/scid mothers. In scid/+ pups of scid/scid mothers, a similar pattern of gradually increasing IgA-coated SFB levels was observed before weaning, followed by a transient decrease right after weaning on day 32. Thereafter, SFB coated with IgA increased to a high stable level. In scid/+ pups of scid/scid mothers, SFB coated with IgA increased to a high level before weaning, with some delay compared to SFB in pups born to scid/+ mothers, and then stabilized at a level comparable to that in scid/+ pups of scid/+ mothers after weaning.

In the ceca and large intestines of scid/+ pups of both scid/scid and scid/+ mothers, much less SFB was coated with sIgA. No difference was observed in scid/+ pups of scid/scid versus scid/+ mothers (ranging from 2 to 12% in the cecum and from 2 to 14% in the large intestine).

DISCUSSION

We have presented data in support of the thesis that maternal IgA and neonatal IgA may be related to the localization of SFB in the guts of neonatal mice. In particular, the ingestion of maternal IgA seems to forestall the colonization of the distal small intestine by SFB, and the active production of intestinal IgA by the pups appears to eliminate the colonizing SFB from the small intestine. The latter shift in SFB colonization has previously been found to occur in conventionally reared mice with a normally developing gut microbiota (34), but here we show that the same shift occurs in monoassociated, immunocompetent pups. We believe ours to be a singular example of the interaction of a normal gut commensal bacterium with the host’s adaptive, mucosal immune system to effect a regulation of the interaction of the microbe with the host's intestinal epithelial cells. A study of a different but analogous interaction, which also may regulate adhesion of a gut commensal microbe, *Bacteroides thetaiotaomicron*, to enterocytes, also utilized GF mice (2). The expression of α1,2-fucosyl glycoconjugates by enterocytes, a ligand for microbial adhesion, required colonization with *B. thetaiotaomicron*. In this case, no role for the host’s adaptive immune system was suggested.

As parts of a common mucosal immune system, mammary and small intestinal tissues of mammals predominantly produce IgA antibodies in their secretions. The sIgA can prevent
the attachment of bacterial and viral pathogens and of entero-
rotoviruses to gut epithelial cells (25, 26, 27). The slgA in the
stomach contents of suckling pups reflects the level of slgA
antibodies in the milk (17, 18). We showed significant differ-
ences in levels of both natural and SFB-specific IgA in the
stomach contents of pups suckling on either monoassociated
scid/scid or scid/+ mothers. FACs analyses of SFB from the
small intestines of suckling pups showed that, before weaning,
these were coated with IgA provided that mothers were scid/+,
no matter what the immune status of the pups (scid/+ or
scid/scid). This preweaning coating correlated with the delayed
colonization of the small intestine with SFB. After weaning,
the high level of IgA production in small intestines from im-
nunocompetent pups correlated with the high level of IgA-
ocated SFB, which resulted in the retreat of SFB from the
small intestine.

Even though only small proportions of maternal milk IgA
and neonatal intestinal IgA seem to be specific for SFB, it is
tempting to suggest that the specific antibodies determine
whether colonization of the small intestine occurs and persists.
SFB attaches to the brush border of the small intestinal epi-
thelial cells by means of a specialized holdfast organelle on the
proximal cell of the linear array of SFB (5, 6, 9). Indeed, it is
tempting to suggest that antibodies interfering with holdfast-
epithelial cell interactions, perhaps via blocking of fucosyl gly-
colipid or glycoprotein interaction, could mediate this anti-
body-bacterial effect. The inhibition of colonization by SFB—
and the subsequent changes in some cellular elements of the
gut—by oral administration of monoclonal anti-SFB was sug-
gested several years ago (39). No supporting evidence has since
appeared regarding the specificity of the blocking antibody, but
the possibility that it may block the holdfast-fucosyl glycolipid
interaction remains a plausible hypothesis. Our observations
seem to offer persuasive evidence that the specific, adaptive
immune systems of nursing dams and developing neonates are
interacting with the SFB in the environment of the isolator to
modulate the timing and localization of gut colonization. Of
course, these immune systems can provide both humoral and
cellular immunity. However, it is very likely that the effect of
nursing, immunocompetent dams is mainly mediated by IgA
antibodies, the major Ig’s in milk. Although exceedingly min-
imal adoptive transfer of cells from fetus to mother has been
shown using FACs analysis (24), we know of no credible evi-
dence that neonatal suckling mice can passively acquire effec-
tive cellular immunity via milk. Foster-nursing experiments
could potentially be used to rule in or out maternal-fetal trans-
fer of immunologic elements that could mediate the effect we
observed—the delay in colonization of the small intestine in
pups born of scid/+ mothers. For instance, we could foster
nurse pups born of +/- mothers on scid/scid mothers and vice
versa using breeding isolates of SFB-monosociated mice.
Certainly, we will attempt these experiments. It is perhaps
relevant that a similar foster-nursing experiment ruled out
prenatal transfer of immune elements with regard to the effects
of nurse dams in forestalling (or not) the active response of
pups to oral reovirus infection (17).

It has been demonstrated that IgA production in the gut is
provided by two different mouse B-cell lineages, B1 and B2
cells (19, 20). B1 cells arise and become competent perinatally,
likely contributing significantly to the active IgA production
that begins around weaning (10, 17, 18). Recently, it has been
found that enteric viruses and gut commensal bacteria stimu-
late both B1- and B2-derived intestinal IgA production, but in
the case of rotaviruses, only B2 cells provide microbe-specific
IgA antibodies (N. Kushner, unpublished data). It will be of
interest to determine whether B1, B2, or both B1 and B2 IgA
can mediate the timing and localization of gut colonization by
SFB.

Although our results argue that maternal IgA and IgA pro-
duced in the neonatal gut are closely correlated to the dynamic
changes in localization of SFB in the guts of mice after birth,
we cannot rule out the possibility that other factors may play
some roles in this process, such as the cellular mucosal immune
responses and/or the changes in SFB-specific colonization re-
ceptors on epithelial cells. Moreover, in conventionally reared
animals, the interactions between indigenous microbiota in the
gut and the host’s immune system may be more complicated
than in monosociated mice. These interactions begin when
the microbiota develop during succession in the neonate and
continue throughout life (1). Local antibacterial immunity may
act synergistically with bacterial antagonism in controlling bac-
terial populations in the intestine (30). Conversely, indigenous
microbiota play an important role in the development and main-
tenance of the normal steady state of GALT (3). Cross
talk among the intestinal microbiota, intestinal epithelium, and
intestinal mucosal immune system is important in forming and
maintaining this dynamic microecosystem (2).

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