Maternal Adaptive Immunity Influences the Intestinal Microflora of Suckling Mice

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ABSTRACT The microflorae in the intestine of breast-fed infants are distinct from those that typically populate the intestine of formula-fed infants. Although the acquisition of passive immunity through breast-feeding may play a critical role in influencing the pattern of bacterial colonization of the gut, the precise mechanisms underlying the differences in the commensal microflorae of breast and formula-fed children have not been established. We hypothesized that the assemblage of commensal microflorae in suckling and weaned mice may be influenced by the maternal adaptive immune system. To test this hypothesis, we analyzed the intestinal microflorae of mice reared in the presence (wild-type) or absence of an intact maternal immune system (T- and B-cell deficient). Several types of bacteria (Lactobacillus, Enterococcus, Clostridium perfringens, Bifidobacterium, and Bacteroides) were isolated and enumerated from both the small and large intestine of 10-, 18-, 25- and 40- to 60-d old mice using selective media. The densities of bacteria were significantly lower in the small intestine of weaned mice that were reared by wild-type (WT) compared with immunodeficient (ID) dams. However, the microflorae were generally more abundant in the large intestine of suckling pups reared by WT compared with ID dams. Our results indicate that intestinal microflorae change throughout the suckling phase of development and that the maternal adaptive immune system influences the pattern and abundance of bacteria within the gut in an age- and site-specific manner. J. Nutr. 134: 2359–2364, 2004.

KEY WORDS: • adaptive immunity • innate immunity • commensal microflora • breast milk

In neonatal mammals, the effector arm of the adaptive immune system is functionally immature as developing lymphocytes undergo positive and negative selection in the thymus (1). During this early phase of development, neonates not only rely on their own innate immune system to help fend off infections but also acquire adaptive and innate immunity through maternal sources (via transplacental routes and breast milk), a process collectively referred to as passive immunity. Passive immunity provides a number of defense factors such as immunoglobulins, lactoferrin, lysozyme, oligosaccharides, cytokines, and chemokines (2,3). Passive immunity may also influence the development of the systemic and mucosal adaptive immune system of neonates (4).

There are profound species differences in the proportions of IgA and IgG in breast milk (2). Dams transfer the humoral portion of the passive immune system either in utero via the placenta or during the postnatal period through breast milk. There is a reciprocal relation between the immunoglobulin isotypes transported by the placenta and secreted by the mammary gland in all mammalian species (5). In some species such as Homo sapiens, IgG is transported via the placenta, and secretory IgA is secreted by the mammary gland during early lactation (6).

In both ruminants and rodents, the IgG provided by breast milk is transferred to the systemic circulation via the neonatal Fc receptor (FcRn),3 which is expressed by enterocytes of the proximal small bowel (7–11). During the suckling phase of development, luminal secretory IgA is provided predominately by breast milk, whereas in postweaned mice, secretory IgA is synthesized by the weanlings own adaptive immune system (10,12). Secretory IgA within the intestinal lumen influences the formation of the bacterial biofilm and presumably the assemblage of commensal and pathogenic bacteria in the gut (13,14).

The small and large bowel house >400 known strains of bacteria, many of which have a symbiotic role in the digestion...
of dietary nutrients (15,16). Furthermore, the intestinal microflora induce the maturation of the mucosal adaptive immune system, thereby providing protection against potential pathogens (17). The exact mechanism by which the consumption of breast milk influences the collection of microflora in the gut is not well understood, but passive immunity is thought to play an important role.

Several studies in mice examined whether the pattern of bacterial assemblage in the gut of breast-fed animals requires an intact maternal adaptive immune system. In 1 model utilizing B cell–deficient (μMT knockout) dams, the patterns of bacterial colonization in the gut of adult mice (>115 d of age) reared by either B cell–deficient or wild-type (WT) mice were indistinguishable when assessed by routine culture techniques (18). A second model compared the pups reared by either SCID (B and T cell–deficient) or WT dams, and evaluated the abundance of segmented filamentous bacteria (SFB) (19). In that study, the SFB load was highest in suckling pups that were breast-fed by SCID compared with WT dams, whereas in postweaned mice, the maternal adaptive immune status had no influence on the abundance of SFB. Interestingly, in postweaned mice, the status of the pups’ own adaptive immune system was more critical because immunodeficient (ID) mice had higher levels of SFB than age-matched WT mice.

In a recent analysis, the pattern of microflora accumulation in the intestine was evaluated by terminal restriction fragment length polymorphisms analysis of 16S rRNA in a polymeric Ig receptor (pIgR)-deficient (absence of secretory IgA and IgM) mouse model (20). The assemblages of bacteria in the terminal ileum of adult pIgR-deficient mice did not differ from those in WT mice. Although this technique is advantageous for surveying a remarkable number of bacterial species, the method is qualitative and therefore incapable of detecting even dramatic differences in the abundance of a specific bacterial species.

Overall, these studies suggest that the maternal adaptive immune system fails to influence either the abundance or type of bacteria in the intestine of adult mice. Thus, we hypothesized that the abundance of 6 prominent species of commensal bacteria in the gut of suckling mice would be influenced by the status of the maternal immune system. Specifically, we compared immunocompetent pups at various ages that were reared by either WT (WT-dam/WT-pup) or ID (ID-dam/WT-pup) dams. Dams that are homozygote knockouts for the recombination-activating (RAG) gene are both B- and T-cell deficient, and therefore have no functional adaptive immune system (10). Overall, we demonstrated that the maternal adaptive immune system influences the pattern of microflora accumulation in the gut in an age and site-specific manner.

MATERIALS AND METHODS

**Mice and breeding scheme.** All animal protocols were preapproved by the UCLA Animal Research Committee (ARC). The MICE used in this study were obtained originally from Jackson Laboratories and subsequently bred and maintained at the UCLA vivarium. They were either C57BL/6J or RAG-1 (−/−) (B6.129S7-RAG-1, former name C57BL6/6J-RAG-1) previously backcrossed 10 generations with C57BL/6J mice. RAG-2 (−/−) mice originally obtained from Dr. E. Rothenberg (California Institute of Technology, Pasadena, CA) and bred within our facility were also used in the study (21). We confirmed the RAG −/− phenotype by the absence of splenic CD3+ staining by fluorescence-activated cell sorting, and serum immunoglobulins by enzyme-linked immunosorbent assay.

Mice were housed in laminar flow racks under specific pathogen-free conditions and provided with autoclaved caging and bedding. They consumed acidified water and irradiated food (Purina Rodent Chow 5001) ad libitum. Pregnant mice were identified and monitored daily until delivery. The day of birth was identified as d 0 of life; mice were culled to a litter size of 6–8 pups and were weaned at 21 d of age. In both experimental groups, we examined a mean of 15 mice at each age (range: 10–20 mice).

To investigate the effect the maternal adaptive immune system has on the microflora of the neonatal intestine, we used a breeding scheme that provided WT pups that were born to and breast-fed by either WT or ID dams. The control group consisted of WT dams that reared WT pups (WT-dam/WT-pup) by mating of C57BL/6 (+/+) females and males. Our experimental group consisted of WT (RAG−/−) dams rearing WT pups (ID-dam/WT-pup) by mating RAG−/− females with C57BL/6 (+/+) males. A targeted disruption of either the RAG-1 or -2 genes results in the absence of an adaptive immune system, including an entire deficiency of T- and B-cell function. Because the RAG phenotype is autosomal recessive, heterozygous mice (RAG−/RAG+) are comparable to WT and have normal T- and B-cell function (21).

**Microbiologic methods.** Mice were sedated and killed by cervical dislocation according to protocols approved by the UCLA ARC. Samples of the small and large intestine were isolated from mice at 4 separate ages: 10, 18, 25, and between 40 and 60 d of age. To improve the reproducibility of our results, samples at all ages contained both intestinal tissue and luminal content. In our experience, separation of the luminal content from intestinal tissue in 10- and 18-d-old mice is particularly difficult to perform consistently. The small and large bowels were carefully removed separately, weighed, and subsequently placed into sterile tubes filled with 5% cysteine and isotonic saline. The samples were immediately homogenized and maintained in either an anaerobic or aerobic environment. Serial dilutions were obtained of the homogenized sample, which were either pour-plated or spread over solidified plates containing selective media.

Selective media were used to enrich the isolation of particular bacteria including Lactobacilli, Bifidobacteria, Enterococci, Bacteroides, and Clostridium perfringens. All cultures were performed as recommended by the manufacture and colony-forming units were enumerated by standard methods (22). Five types of media were used and prepared as follows: 1) MRS agar (Oxoid) was used for the enrichment of Lactobacillus; 2) the same agar enriched with 5% sheep blood (Quelab), 0.2% LiCl, 0.3% Na propionate, and 0.05% cysteine was used to isolate primarily Bifidobacteria; 3) Slanetz and Bartley agar (Oxoid) was used to enrich for Enterococcus; 4) perfringens agar (OPSP) supplemented with ASR76 and BSR77 (Oxoid) was used to enrich for Clostridium perfringens; and 5) Wilkins Chalgren agar enriched with SR108 (Oxoid) and 5% horse blood (Quelab) was used to improve the isolation of Bacteroides. The Slanetz and Bartley plates were incubated aerobically for 48 h; all other plates were anaerobically incubated in gas pack jars (BBL) with CO2, generating kits (Becton Dickinson) at 37°C for 48 h.

**Statistical analysis.** We compared mean standardized bacterial load across the 2 experimental conditions and 4 time periods using a 2 × 4 factorial ANOVA. Statistical significance was assessed using post-hoc t tests and the Tukey-Fisher criterion using the ANOVA model. All data presented are means ± SE.

**RESULTS**

Changes in the bacterial flora of the small and large bowel during development. The bacterial assemblage in the small and large intestine varied by age in pups reared by WT dams (WT-dam/WT-pup) (Figs. 1 and 2). The most dramatic age-dependent differences were in the small intestine between 10 and 18 d of age, when all species except C. perfringens changed significantly (Fig. 1). The number of Lactobacillus, Bifidobacteria, and C. perfringens colonies declined 90% between 10 and 40 to 60 d of age. Bacteroides had the most dramatic decline (3 orders of magnitude) of all species isolated from the small intestine between the suckling and weaned periods of development. Conversely, Enterococcus was the only species whose abundance increased throughout the first 3 wk of life, after which the levels began to decline. Finally, at every age, Lac-
tobacillus was at least 100-fold more abundant than any other bacterial species.

The large intestine had a higher overall number of bacterial species that fluctuated less with age compared with the small intestine (Figs. 1 and 2). Lactobacillus, Bifidobacteria and Bacteroides levels all declined similarly between 10 and 18 d and stabilized thereafter (Fig. 2). During the suckling period of development, the load of Bacteroides in the large intestine declined by 2 orders of magnitude and decreased >90% in the small intestine. In contrast, with the exception of a small decline in the abundance of Enterococcus between 10 and 18 d of age, the C. perfringens and Enterococcus species did not show dramatic developmental differences in the large bowel. These 2 species were the least abundant bacteria isolated from the large intestine, whereas Lactobacillus species were the most plentiful.

**Passive immunity alters the quantity bacteria in the small intestine.** In the small intestine of 40- to 60-d-old mice, the quantity of Lactobacillus, C. perfringens and Bifidobacteria were 90% lower in mice reared by WT compared with ID dams (Fig. 3A, B, C; P < 0.005). The abundance of these bacterial species at other ages was not affected by the maternal immune status. In contrast, at 10 and 40–60 d of age, Enterococcus was significantly more abundant in pups reared by ID compared with WT dams, whereas the opposite results were obtained at 18 and 25 d of age (Fig. 3E; P < 0.005). By 10 and 18 d, mice reared by WT dams had 100-fold more Bacteroides than age-

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**FIGURE 1** Developmental changes in Lactobacillus, Bifidobacteria, Bacteroides, Enterococcus, and C. perfringens composition of the small intestine of mice reared by WT dams at various ages. Data are presented as the mean log10 colony forming units × 10⁻³/g specimen ± SD (calculated only for positive cultures). For each bacteria, age group means without a common letter differ, P < 0.05.

**FIGURE 2** Developmental changes in Lactobacillus, Bifidobacteria, Bacteroides, Enterococcus, and C. perfringens composition of the large intestine of mice reared by WT dams at various ages. Data are presented as the mean log10 colony forming units × 10⁻³/g specimen ± SD (calculated only for positive cultures). For each bacteria, age group means without a common letter differ, P < 0.05.

**FIGURE 3** Passive immunity influences the microflora of the small intestine of WT mouse pups reared by WT or ID dams at various ages. (A) Lactobacillus, (B) Bifidobacteria, (C) C. perfringens, (D) Bacteroides, (E) Enterococcus. Data are presented as the mean log10 colony forming units × 10⁻³/g specimen ± SD (calculated only for positive cultures). *Means for groups at that age differ, P < 0.05.
matched pups reared by ID dams (Fig. 3D; \( P < 0.005 \)). In contrast, maternal immune status did not affect the abundance of *Bacteroides* in the small bowel of mice between the ages of 25 and 40 to 60 d of age.

**Passive immunity alters the quantity bacteria in the large intestine.** The trends in the large intestine were quite distinct from the findings in the small intestine (Figs. 3 and 4). Only *C. perfringens* and *Bifidobacteria* demonstrated similar trends in both tissues. The abundance of *Lactobacillus* in the large intestine was influenced by the maternal immune status only at 10 and 40–60 d of age (Fig. 4A, \( P < 0.005 \)). At 10 d of age, pups reared by WT dams had fewer *Lactobacillus*, whereas at 40–60 d of age, mice from the same group had higher levels.

Interestingly, the quantities of *Enterococcus* and *Bifidobacteria* were not influenced by the maternal immune status at any age examined (Fig. 4B and E). In contrast, the quantity of *C. perfringens* was an order of magnitude higher in 10-d-old pups reared by WT dams compared with similar aged pups reared by ID dams (Fig. 4C; \( P < 0.005 \)). There were no differences at any other age. Unlike all previous groups, accumulation of *Bacteroides* in the large bowel was >1000-fold higher at all ages in mice reared by WT dams compared with similarly aged mice previously reared by an ID female (Fig. 4D; \( P < 0.001 \)).

We analyzed how the incremental increase in intestinal weight influenced bacterial concentrations as the mice aged. When compared to 10 day old pups, the relative weight of the small bowel (log10 \( \pm \) SEM) of mice in the WT-dam/WT-pup group was 0.47 \( \pm \) 0.06 at 18 d, 0.72 \( \pm \) 0.05 at 25 d, and 0.89 \( \pm \) 0.04 at 40 d. Similar results (not significantly different) were obtained in ID-dam/WT-pup mice. In contrast, when compared to the youngest age group, the relative weight of the large bowel of pups from the WT-dam/WT-pup group was 0.25 \( \pm \) 0.01 at 18 d, 0.66 \( \pm \) 0.07 at 25 d, and 0.94 \( \pm \) 0.06 at 40 d. Interestingly, while similar results were obtained during the first two ages of ID-dam/WT-pup group, the large intestines of these mice were significantly larger at both 25 (0.8 \( \pm \) 0.05) and 40 d of age (1.04 \( \pm \) 0.07). Therefore, while our data confirm a general decline in the density of microflora per gram of small or large bowel, there was an incremental increase (by as much as 11-fold) in the mass of the intestine as the mice aged.

**DISCUSSION**

Many epidemiologic studies demonstrated the benefit of breast- over formula-feeding and led the American Academy of Pediatrics and the WHO to recommend breast milk as the gold standard of infant nutrition in the vast majority of clinical settings (23). Specifically, the incidence of neonatal sepsis and necrotizing enterocolitis (NEC) are significantly reduced in breast-fed compared with formula-fed infants (24,25). However, other feeding practices were implicated in the pathogenesis of disorders such as NEC (26). Breast-feeding also protects against numerous other infections such as otitis media, upper and lower respiratory tract, intestinal and urinary tract infections, while reducing the risk of developing many immunemediated disorders including celiac disease, inflammatory bowel disease, and diabetes mellitus (27–29).

Despite the many benefits of breast-feeding, the exact mechanism of how breast milk exerts its influence has not been established. Passive immunity is provided by the maternal innate and adaptive immune systems. Among the components of adaptive immunity, immunoglobulins (IgG and IgA) have been most frequently implicated as the main factor that protects neonates against various disorders (30–34). Secretory immunoglobulins specifically were suggested to play a role in protecting against adherence of enteric microflora to the epithelial layer. However, in several murine models, the role of secretory IgA in influencing the bacterial microflora of the gut was inconclusive (18–20). Specifically, when luminal IgA was eliminated in 2 knockout models (\( \mu MT \) and pIgR), the bacterial composition of the intestine in both models was indistinguishable from that of controls (18,20). The design of these studies had several limitations that minimized the conclusions that could be drawn in understanding how secretory IgA
influences the enteric microflora. Both studies limited their evaluation to adult animals (>4 mo of age), an age at which the influence of passive immunity would be less apparent. Finally, the microflora of plgR knockout mice were evaluated using terminal restriction fragment length polymorphism analysis of bacteria-specific 16S rRNA (20). Although this technique is powerful and is capable of detecting species that are not possible to culture, determining differences in the abundance of specific species is beyond its capabilities. We examined whether the maternal adaptive immune system, and consequently the presence or absence of the adaptive component of passive immunity, altered the abundance of key intestinal microflora during the suckling and weaned periods of development. Previous studies used SCID mice but we chose RAG knockout mice because SCID mice become "leaky" with respect to maintaining their humoral immunodeficiency as they age (19,35). Overall, our study found that of the 6 bacterial species analyzed, Bacteroides assemblage in both the large and small bowel was modified as a consequence of the maturation of the maternal immune system. Specifically, in the small intestine, the level of Bacteroides was >100-fold higher during the suckling phase of development in pups that were reared by WT dams (Fig. 3D). A similar difference in the content of Bacteroides was also seen in the large intestine at all ages examined (Fig. 4D). Many sub-strains of Bacteroides were reported, some of which have been implicated in the activation of the developing mucosal immune system. Our study suggests that a component of the maternal adaptive immune system either directly or indirectly promotes the expansion of Bacteroides. A corollary hypothesis in this study was that an intact maternal immune system would likely promote the abundance of commensal flora in the gut of breast-fed offspring; these are considered more beneficial and are commonly known as probiotic organisms (36,37). Within this context, it was also suggested that luminal secretory IgA may play an important role in preventing the accumulation of allochthonous or pathogenic bacteria, without necessarily affecting autochthonous or indigenous bacteria. Compared with their formula-fed counterparts, breast-fed infants generally have a relative abundance of Lactobacillus and Bifidobacteria, 2 bacterial strains that are considered classic probiotic organisms. In our study, the concentrations of Lactobacillus and Bifidobacteria in the small intestine of 40- to 60-d-old mice, were both more abundant in mice previously reared by ID dams (Figs. 3A and B). In contrast, Lactobacillus levels were higher in the large intestine of 40- to 60-d-old mice reared by WT dams (Figs. 4A). These data suggest that maternal immune status influences the pattern of the intestinal microflora in the nursing animal in a complex manner that depends on age and region of the intestine and is specific to certain bacterial species. Breast milk oligosaccharides may promote the selective proliferation of Bifidobacteria and Lactobacillus growth, and formulas supplemented with various types of oligosaccharides were marketed as "prebiotic" (38,39). We did not demonstrate in this murine model that the maternal adaptive immune system favored the general expansion of the probiotic-type of microflora. Taken together, the data suggest that some other component of breast milk, such as oligosaccharides, may promote the expansion of probiotic organisms. The RAG experimental system that we employed in the study does not precisely mimic differences between humans that are breast- vs. formula-fed. ID RAG dams still retain an active innate immune system that produces soluble factors that enter breast milk and are not present in commercial formulas. Lactoferrin, for example, was shown to influence the formation of the bacterial biofilm, and thereby alter the pattern of commensal assemblage (40). Chemokines and cytokines are also selectively present in large quantities in breast milk, and because they represent a conduit between the innate and adaptive immune system, it remains to be established whether their relative abundance in the breast milk of RAG-deficient and WT dams differs substantially (41). Overall, the functional innate immune system of the RAG-deficient dams is likely to continue to influence the pattern of microflora accrual in the neonatal gut. The developmental trends of bacterial colonization of suckling and weaned mice reared by WT dams did not differ between the small and large intestine. Overall, for most bacterial species, there were general declines in the bacterial densities with aging (Figs. 1 and 2). In the small intestine, this was true for all types of bacteria except C. perfringens. On the other hand, in the large intestine, all bacterial species decreased in concentration with the exception of C. perfringens and Enterococcus. Interestingly, a common trend throughout most groups was a decline in the abundance of bacterial species between 10 and 18 d of age. This decline was followed by an increase in bacteria concentration between 18 and 25 d of age, and another decline between 25 and 40 to 60 d of age. The increase between 18 and 25 d may be due to the process of weaning because pups begin consuming solid foods by 15 d and are fully weaned by 21 d of age. Interestingly, the mucosal adaptive immune system of suckling rodents begins to mature during this phase of development, when lymphocytes begin populating the lamina propria and intraepithelial compartments (4). The contribution that the animal’s own adaptive immune system has in controlling the assemblage of microflora in the gut was not evaluated in the current study. Our study contains several important limitations that we will address briefly. Although we initially attempted to quantify the mucosal adherent flora, we eventually decided to focus on determining the total bacterial content of the small and large bowel. We found that younger pups had very fragile intestines that made it practically impossible to consistently separate luminal contents from adherent bacteria, while still isolating the anaerobic flora. Subsequent experiments are warranted to further investigate the importance of the infant’s own adaptive immune system in establishing the normal intestinal microflora in the small and large intestine. A third limitation of this study is that we focused only on a small portion (6) of the many bacterial species that occupy the mouse intestine. Moreover, advanced methodologies that would confirm the precise identities of these microbes were beyond the scope of this current study. New methods, such as oligonucleotide fingerprinting of rRNA genes, are independent of standard bacterial isolation and culturing techniques and should allow for a more comprehensive analysis of how maternal immune status influences the pattern of commensal colonization (42). LITERATURE CITED


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