

# Influences of microbiota on intestinal immune system development<sup>1-3</sup>

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**ABSTRACT** The normal colonization of the mammalian intestine with commensal microbes is hypothesized to drive the development of the humoral and cellular mucosal immune systems during neonatal life and to maintain the physiologically normal steady state of inflammation in the gut throughout life. Neonatal conventionally reared mice and germ-free, deliberately colonized adult mice (gnotobiotic mice) were used to examine the efficacy of certain intestinal microbes. *Am J Clin Nutr* 1999;69(suppl):1046S–51S.

**KEY WORDS** Gut flora, intestinal microbes, mucosal immune system, development of immunity, enteric viruses, bacterial gut commensals, colonization resistance, humoral mucosal immunity, cellular mucosal immunity, mice

## INTRODUCTION

The main thesis of this article is that commensal gut bacteria and enteric viruses stimulate the normal development of the humoral and cellular mucosal immune systems. These interactions with environmental antigens may benefit and activate specific and adaptive, natural (ie, not deliberately stimulated) and semispecific, and aspecific elements of the mucosal immune systems. A corollary is that these interactions between the mucosal immune systems and enteric microbes, presented in ever-changing and novel patterns, maintain the physiologically normal state of inflammation or activation of gut-associated lymphoid tissue throughout life.

It has been known for decades that gut commensal microbes colonizing neonatal mammals effect the activation and development of the systemic immune system, especially by increasing circulating specific and natural antimicrobial antibodies (1–6). Generally, porcine and murine germ-free neonates and their young-adult counterparts have been compared after deliberate colonization with defined gut enteric bacteria. These gnotobiotic animals were in turn compared with their counterparts that were naturally or deliberately colonized with the still incompletely defined, normal gut flora (conventional animals). Generally, colonization with a single gut commensal or a simple mixture was not as effective at driving the development of the natural immune system as was conventionalization of animals with the entire, uncharacterized gut flora.

We chose to focus on the effects of gut microbes in the development of the mucosal immune system, especially that of the gastrointestinal tract. Generally, the gut-associated lymphoid tissue can be divided arbitrarily into 3 compartments, each with its

own conspicuous and characteristic immune elements and reactions: 1) Peyer patches (PPs), which are organized lymphoid tissues in the wall of the small intestine that contain B lymphoid follicles and interfollicular populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, many with the propensity to recirculate to and selectively lodge in mucosal tissue; 2) gut lamina propria, which is the meshwork of connective tissue underlying the gut epithelium that contains a broad spectrum of myeloid and lymphoid cells, especially immunoglobulin (Ig) A plasmablasts, CD4<sup>+</sup> T cells, dendritic cells, and mast cells; and 3) intraepithelial leukocyte spaces, which are the spaces between intestinal epithelial cells and above the basement membrane that are populated by a variety of small, round cells, especially natural killer cells and many CD8<sup>+</sup> T cell subsets (7, 8). The status of these 3 compartments with respect to the numbers and activation states of their conspicuous cellular elements seems to depend on stimulation by gut microbial antigens.

## GENERAL PRINCIPLES BASED ON NEONATAL AND GERM-FREE MOUSE MODELS

The physiologically normal activated state of B cell follicles in PPs, displaying continuous germinal center (GC) reactions, depends on chronic and novel gut mucosal stimulation by enteric antigens. Unlike the peripheral lymph nodes and spleens of conventional mice, the PPs display chronically activated (secondary) rather than quiescent (primary) B cell follicles (9, 10). Of particular interest is that the GC reactions of PPs preferentially generate IgA-committed, antigen-specific B cells—effector and memory—in many mammals, such as rabbits, rats, mice, and humans (7, 10–14).

We first chose to use orally applied reovirus type 1 to perturb the quiescent B cell follicles in the PPs of germ-free mice (13). Reovirus type 1 selectively sorbs to M cells of follicle-associated epithelium overlying the lymphoid compartment of PPs and is delivered to and stimulates these elements (15). We found that 1) PP GC reactions waxed and waned over ≈4 wk, reaching a

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maximum of GC B blasts, many of which were IgA<sup>+</sup>, at ≈14 d post-immunization, and 2) a secondary oral challenge with reovirus resulted in a considerably reduced perturbation of GC reactions and much less output of specific IgA antibodies by mucosal tissue than was observed after the primary infection (13). We used organ fragment cultures of PPs and small intestine to monitor the gut mucosal IgA response (16). The conclusions were that PP GC reactions were transient and that a successful secretory IgA response attenuated the stimulation by secondary mucosal challenge. This observation was the reverse of what was observed in primary and secondary footpad inoculation of reovirus, in which lymph node tissue showed GC reactions that increased in magnitude—as did output of IgG antibodies—on successive stimulation. Thus, successful mucosal immune responses seemed to control and down-regulate subsequent mucosal responses. The gut mucosal reovirus infection itself was resolved within 12–14 d as expected. These observations raised the question of whether chronic colonization or infection of the gut lumen would continuously drive mucosal IgA responses or would be down-regulated by the initial responses, as was observed in successive oral reovirus challenges.

To address this issue, as well as the perennial question of whether mammals have mucosal immune responses to their autochthonous, commensal microbes, we deliberately colonized germ-free mice with a distinctive commensal microbe, *Morganella morganii* (17). *M. morganii* is a Gram-negative, facultative anaerobe that contains a distinctive phosphocholine determinant attached to its lipopolysaccharide (MN Young, JC Richard, unpublished observations, 1994). We found that 1) GC reactions in PPs waxed and waned with the same kinetics as for oral reovirus infection, 2) the bacteria remained in the gut at high density ( $10^8$ – $10^9$ /g feces) for ≥1 y, 3) the specific IgA plasmablasts in gut lamina propria and IgA memory cells in PPs gradually declined after reaching their maximal number at 4–10 wk post-immunization to a low but effective plateau for 1 y post-immunization, 4) the translocating bacteria eventually disappeared from the spleen and mesenteric lymph nodes, and 5) the remaining bacteria in the gut were continuously coated with IgA (18). Thus, it appears that mice do respond to colonization with commensal microbes, but that this response is self-limiting and precludes continuation of GC reactions and further new stimulation of effector IgA plasmablasts in new GC reactions. The basis for maintenance of specific memory IgA B cells and the stimuli that resupply the gut lamina propria with specific IgA plasma cells remains an enigma.

### Is there a delay in the development of neonatal humoral mucosal immune responsiveness? What regulates the neonatal development of active mucosal immunity against gut microbial antigens?

It has long been known that neonatal mice show delayed rises in antigen-specific B cells to the normal adult frequencies that vary depending on the determinant, especially bacterial determinants such as  $\alpha 1 \rightarrow 3$  dextran,  $\alpha 1 \rightarrow 6$  dextran,  $\beta 2 \rightarrow 1$  fructosan (inulin), phosphocholine, and  $\beta$ -galactosyl (19, 20). These differentially delayed rises have been attributed to genetically pre-programmed recombinational events (19), perhaps contributing to an idio- or anti-idio- type network that modulates development of the diverse antibody specificities (21), and also to the natural stimulation of the neonatal immune system by environmental antigens, particularly gut microbial colonizers (20, 22). It

is also known that there is a paucity of IgA plasmablasts in the gut lamina propria of newborn mice (23), and that specific antigen-sensitive B cells in PPs committed to IgA (IgA memory cells) reactive against inulin, phosphocholine, and  $\beta$ -galactosyl determinants take weeks after birth to rise to adult proportions. The proportions of antigen-sensitive cells among PP cells at 5 and 8–11 wk after birth are shown in **Table 1** (R Shahin, JJ Cebra, ER Cebra, unpublished observations, 1983). We assessed Ig-isotype potential by using the splenic fragment assay of Klinman et al (19). Note that only 9% of the clonal precursors found at 5 wk of age were committed to exclusive IgA production, whereas at 8–11 wk, 47% were IgA memory cells. At the time we speculated that a change in gut flora accompanying weaning, a decline in passively acquired maternal antibodies to inulin, or both could result in an increased natural stimulation of inulin-reactive cells at 3–5 wk of age (20).

Recently, to address whether neonatal mice have an underdeveloped or competent humoral mucosal immune system, we infected 10-d-old pups orally and intragastrically with reovirus type 1, a potent stimulator of both humoral and cellular mucosal immune responses in adults (15) that is absent from our specific pathogen-free mouse colonies. Thus, mice in our colonies normally have no circulating or mucosal antibodies reactive with or against reovirus. Reovirus type 1 causes a transient intestinal infection without clinical symptoms in both 10-d-old and adult mice. By using the method of PP and small-intestine organ fragment culturing (16), we found that pups displayed prompt, specific IgA antibody responses in 3–5 d that were of a magnitude

**TABLE 1**

Immunoglobulin isotypes expressed by clones from  $\beta$ -galactosyl-specific, phosphocholine-specific, and  $\beta 2 \rightarrow 1$  fructosyl-specific Peyer patch B cells taken 5 wk and 8–11 wk after birth<sup>1</sup>

Isotype	Age of donor	
	5 wk	8–11 wk
	n	
A only <sup>2</sup>	3 (9)	21 (47)
M only	23	14
A + M	2	3
A + G3	0	1
A + G2 + G3 + M	0	2
A + G1 + M	1	2
A + G3 + M	0	1
Some A <sup>3</sup>	6 (18)	30 (66)
G1 + A	3	1
G1 + M	3	1
Total clones	32	45

<sup>1</sup>Peyer patches were taken from conventionally reared but not deliberately immunized young mice. Cell suspensions were transferred into hemocyanin-primed, lethally X-irradiated, syngenic mice at a limiting dilution. At 24 h after transfer, spleens were harvested from recipients, diced into 50 fragments, and cultured at high oxygen concentrations in wells with a mixture of hemocyanins conjugated to either  $\beta$ -galactosyl, phosphocholine, or  $\beta 2 \rightarrow 1$  fructosyl. After 8–10 d of culture, supernatants were harvested and assayed by radioimmunoassay for antibodies to these 3 determinants and for the immunoglobulin isotype of these antibodies. Results are for statistically clonal cultures.

<sup>2</sup>Percentage of clonal precursors committed to exclusive immunoglobulin A production in parentheses.

<sup>3</sup>Among the various isotypes of antibodies produced by the culture, some immunoglobulin A antibodies were detected; percentage in parentheses.

similar to responses found in young adult hosts (24). We also found that although noninfected control pups made neither specific antibody nor natural IgA, immunized pups had  $\approx 10$ – $20$  times more natural IgA than specific IgA antireovirus (Table 2). Apparently, 15-d-old pups are competent to develop natural IgA responses, but do not do so until 22 d of age unless challenged with an infectious, enteric virus. We use the term natural IgA to designate all IgA produced that cannot bind to our sorbed antigen preparations. Of course, some of this natural IgA may be specific for antigens of the infecting or colonizing microbe that are not adequately represented in our sorbed antigen preparations. However, the production of most natural IgA appears to be stimulated, not necessarily specifically, by the presence of members of the normal gut flora.

We devised an approach to discern possible effects of the immune system of both birth and nurse mothers on the development of the neonatal mucosal immune system, especially on the expression of natural IgA (25). We reciprocally crossed immunocompetent mice with coisogenic severe combined immunodeficient (SCID) mice. The homozygous SCID defect prevents the expression of any form of specific, adaptive immune response (26), whereas the heterozygous  $F_1$  mice (SCID/+) are fully immunocompetent. Thus, the only difference between the 2 groups of  $F_1$  mice was whether they were born of SCID or immunocompetent mothers. We switched  $F_1$  mice at birth so that mice born of SCID mothers were nursed by immunocompetent dams and vice versa. We made several principal findings as follows: 1)  $F_1$  pups born to and nursed by SCID mothers showed a precocious rise in natural IgA production in PPs and small intestine at day 15–16 after birth, whereas such expression of IgA was delayed until at least day 22–25 in pups born to immunocompetent dams; 2) this difference between the 2 groups of  $F_1$  pups was reflected by IgA-secreting cells from gut PPs and lamina propria and by GC reactions in the B cell follicles of PPs: by day 16, pups born of SCID mothers already had  $\approx 200$  IgA antibody-secreting cells (ASC)/ $10^6$  dispersed small round cells in PPs, 2000 IgA ASC/ $10^6$  in lamina propria, and robust GC reactions in PPs, whereas pups born of immunocompetent dams had negligible IgA ASC and no evidence of GC reactions; 3) the stomach contents of  $F_1$  pups of immunocompetent mothers were exceedingly rich in suckled, maternal IgA, whereas maternal IgA was absent from stomachs of pups born of SCID mothers; and 4) the bacteria isolated from the guts of pups born to and nursed by immunocompetent dams were coated with IgA at an early age (by day 10), whereas bacteria from pups of SCID dams were

uncoated initially but, beginning on day 16 after birth, gradually acquired an IgA coating, likely as a result of the active production of endogenous IgA (25). We suggest that maternal IgA antibodies, by coating gut commensal bacteria in neonatal intestine, shield or block the neonatal immune system from antibody stimulation and delay the active development of natural IgA responses to these environmental antigens.

To test whether expression of mucosal antibody responses by the nurse mothers could interfere with the development of active mucosal immunity by the neonates, we used a nonenvironmental antigenic stimulus, reovirus (24). Immunocompetent female mice were either infected orally with reovirus 2 wk before mating with SCID males or not. The  $F_1$  pups of these matings were challenged at day 10 with reovirus, along with  $F_1$  pups of the reciprocal cross: SCID mothers and immunocompetent fathers. In some experiments, the newborn litters were swapped and foster nursed. All permutations of the 3 types of  $F_1$  litters and 3 types of nurse mothers were tested for specific gut mucosal IgA responses to reovirus and increase in production of natural IgA by their gut tissues (24).

Our findings were as follows. 1)  $F_1$  neonates from SCID or immunocompetent mothers responded equally well with gut mucosal IgM and IgA antibodies against reovirus, and both groups of pups expressed similar rises in natural IgA. 2) These results from organ fragment cultures were reflected by analyses for IgA ASC in gut lamina propria: specific IgA ASC rose to  $\approx 10^2/10^6$  lymphocytes in PPs and mesenteric lymph nodes of both groups of  $F_1$  neonates, whereas they remained negligible in nonimmunized controls; total (natural) IgA ASC also rose to similar levels,  $8$ – $9 \times 10^2/10^6$ , in both groups but were negligible in control pups of immunocompetent dams and were higher,  $1.5 \times 10^2/10^6$ , in control pups of SCID dams. 3) When litters were swapped, the responsiveness of the pups to oral reovirus challenge was almost always the same: the immunologic status of the birth dams was irrelevant, whether SCID, immunocompetent, or immunocompetent orally immunized with reovirus. However, the immunologic status of the nurse dams was critical in determining the outcome of oral challenge of the pups: if the nurse mothers had been orally immunized, then pups did not make an active specific IgA antibody response to reovirus. Generally, the expression of a rise in natural IgA followed the specific response to reovirus. The single exceptional permutation was the case of pups born of SCID mothers but nursed by reovirus immune dams. These pups showed no expression of gut mucosal antibodies against reovirus after an oral challenge with reovirus but

**TABLE 2**

The rise in natural immunoglobulin (Ig) A and microbial-specific Ig A antibody (Ab) is differentially stimulated by viral infection of conventionally reared (CNV) neonates or segmented filamentous bacteria (SFB) colonization of formerly germ-free (GF) adult mice

	Time postimmunization <sup>1</sup>	Specific IgA Ab output <sup>2</sup>	Total IgA output <sup>3</sup>	Specific IgA Abs
	d	$\mu\text{g/L}$	$\mu\text{g/L}$	%
Reovirus type I in 10-d-old immunocompetent neonate <sup>4</sup>	6	270	2500	10.8
SFB in GF immunocompetent adult				
Experiment 1	85	1.6	2060	0.08
Experiment 2	14	21	835	2.5

<sup>1</sup>Day post-immunization at which percentage specific IgA Ab was greatest.

<sup>2</sup>Output of IgA (specific or total) in 1.0 mL culture of Peyer patch or small intestine.

<sup>3</sup>Typical values for output of total IgA from PP or SI fragment cultures from CNV adult mice are 2000–5000  $\mu\text{g/L}$ .

<sup>4</sup>See references 24 and 25.

did show a significant rise in natural IgA. We are now investigating this exception.

Recently, we sought a procedure by which neonates born of mucosally immune mothers could benefit from passively acquired, suckled maternal IgA antibodies while still being actively immunized via the mucosal route. We found that live reovirus type 1, a protective vaccine against reovirus type 3 challenge (27), can be encapsulated by using aqueous interactions between spermine and alginate (28). These small ( $\approx 5 \mu\text{m}$ ) capsules can be given orally and circumvent the neutralization by suckled maternal antibodies to stimulate active immune responses by neonatal mice (28).

### **Can environmental antigens, particularly members of the gut commensal flora, that drive development of the mucosal immune system, be identified?**

Schaedler et al (29–31) pioneered the identification of members of the indigenous gut flora of mice, the development of this flora in neonates, and the colonization of germ-free mice with these particular bacteria. Generally, the lactobacilli, enterococci, and slow-lactose fermenting coliforms identified were facultative anaerobes or fusiforms and clostridia that could be cultivated *in vitro* under anaerobic conditions. Crabbe et al (32) and Moreau et al (33) used mixtures of such isolated commensals to colonize germ-free mice and followed the appearance of IgA plasmacytes in gut lamina propria. Germ-free mice have a paucity of IgA plasmablasts in their gut lamina propria (34). After gut colonization with either uncharacterized gut fecal flora (32) or mixtures of the gut commensals identified by Schaedler et al (29, 31), appreciable numbers of gut IgA plasma cells developed in formerly germ-free mice. These cells reached nearly two-thirds of the normal level observed in conventional mice (33). Usually, monoassociation with a single gut bacterial species was much less effective in inducing this development of IgA plasmablasts in the gut than were various mixtures.

In the past decade we learned that major contributors to the gut flora of mice, and of many animals, are obligate anaerobes that have not yet been cultured *in vitro* (35). Indeed, Joseph Leidy (36) described a dominant microbial type in 1849, segmented filamentous bacteria (SFB), which he found first in the midgut of termites. This SFB type was tentatively named arthromitis, and relatives have been found in the chicken, rat, and mouse (37). This Gram-positive, segmented obligate anaerobe is spore forming and was recognized as a dominant gut microbe of mice by Savage et al (35, 38). Klaasen et al (39) recently isolated SFB by treating fecal material with organic solvents to kill vegetative organisms and administering a series of ever-weaker inocula into germ-free mice. Snel et al (37) used 16S ribosomal RNA sequence analysis to position the SFB of several vertebrates within the cluster of clostridial species. Perhaps of greater relevance to this topic, Klaasen et al (40) found that monoassociation of mice with spores of this single microbial species results in a profound stimulus for the development of IgA ASC in the gut lamina propria. Recently, we collaborated with this group (H Snel, F Poelma, P Heidt, G Talham, JJ Cebra, unpublished observations, 1997–1998) to distinguish specific from polyclonal (natural) IgA plasmablast development driven by colonization of germ-free mice with SFB and to evaluate how effective this stimulus was compared with normal expression of natural IgA by conventional mice. Our findings were that 1) GC reactions occurred in PPs by 14–21 d postimmunization and that

these gradually waned over 100 d of colonization, 2) natural IgA output by gut-associated lymphoid tissue fragment culture followed GC reactions in PPs and reached levels of 34–87% of that found from gut-associated lymphoid tissue of conventional mice, and 3) specific IgA antibodies did develop but never exceeded 2.5% of the total natural IgA output (Table 2). Thus, it seems that SFB, which appear in rats and mice only around the time of weaning (41, 42) may be a major stimulus of the development of the natural mucosal IgA system. These SFB grow out in both the small and large intestine at around the time of weaning and become the major intestinal microbe. Thereafter, they retreat from the small intestine to remain dominant in cecal fluid and the large intestine. Because this shift in population occurs in formerly germ-free immunocompetent mice, but not in formerly germ-free SCID mice, we postulate that the host immune response may play a role in the retreat of SFB from the small intestine. Perhaps of relevance to our findings is that SFB attach to the apical surface of intestinal epithelial cells via an organelle that is the first segment (holdfast) in the small intestine (38). We speculate that 1) luminal antibodies against attachment factors may prevent attachment such that surviving organisms in cecal fluid and the colon are largely luminal, and 2) the attachment, without breach, to epithelial cells may stimulate their production of cytokines that may contribute to the activation of the specific and nonspecific elements of the mucosal immune system.

### **DO MEMBERS OF THE GUT COMMENSAL FLORA ACTIVATE CELLULAR ELEMENTS OF THE GUT MUCOSAL IMMUNE SYSTEM?**

For brevity, this discussion will be confined to SFB colonization. Generally, T lymphocytes and natural killer cells in the gut mucosa are normally in a much more activated state than similar cells found in peripheral lymph nodes and the spleen. For instance, most peripheral CD4<sup>+</sup> T cells express the CD45RB<sup>high</sup> phenotype, indicative of resting or unprimed helper T cells. Also, CD8<sup>+</sup> T cells in the periphery are ordinarily not preactivated to kill target cells to which they are coupled. However, PP CD4<sup>+</sup> T cells include a majority of CD45RB<sup>low</sup> T cells which are preprimed, or activated. The intraepithelial leukocyte compartment also contains highly activated natural killer cells as indicated by their target cell range (CF Cuff, JJ Cebra, unpublished observations, 1996) and “constitutive” CD8<sup>+</sup> cytotoxic cells that will kill targets to which they are coupled, without prior *in vitro* activation (43). The constitutive killers are usually detected by a technique called facilitated cytotoxicity, in which the Fc-receptor-bearing targets are coated with IgG monoclonal antibodies reactive with the components of T cell receptors and can be coupled with any T cell positive for T cell receptors (44).

Both the CD4<sup>+</sup> cells in PPs and the CD8<sup>+</sup> or natural killer cells in intraepithelial leukocyte spaces of germ-free animals are quiescent. We have found that colonization with SFB causes a gradual shift, over several months, of CD4<sup>+</sup> PP cells from CD45RB<sup>high</sup> to a majority of CD45RB<sup>low</sup> cells (8). We do not yet know about changes in functional potential of these cells. Similarly, these SFB colonized BALB/c mice show a rise in constitutive cytotoxicity among the CD8<sup>+</sup> T cells of the intraepithelial leukocyte spaces (45, 46). We have confirmed this activation by SFB in formerly germ-free C3H mice over 70 d of colonization and have also found a profound activation of the natural killer cells in the intraepithelial leukocyte compartment (47). Thus,



these single gut commensal bacteria, SFB, seem capable of activating both humoral and cellular mucosal immune systems in a nonspecific way.

### CAN COLONIZATION BY GUT COMMENSAL BACTERIA PROVIDE PRACTICAL HOST RESISTANCE AGAINST FRANK OR OPPORTUNISTIC PATHOGENS?

This subject has an enormous body of literature (48). However, I will focus on a few examples of colonization resistance prompted by gut commensals, ie, the upward alteration of the number of orally encountered bacteria required to cause clinical symptoms and pathologic changes. Garland et al (41) showed a clear relation between the outgrowth of SFB in the intestines of neonatal rats and their ability to withstand an otherwise lethal oral dose of *Salmonella enteritidis*. Zachar and Savage (49) showed that colonization of germ-free mice with 2 members of the indigenous flora, bacteroides and clostridium, would greatly increase colonization resistance against the opportunistic oral pathogen *Listeria monocytogenes*.

The oral dose of *L. monocytogenes* required to kill germ-free mice is  $1-5 \times 10^2$  (49), whereas conventional mice survive oral doses of  $10^8-10^9$  (50). Obviously, colonization resistance conferred by the normal gut flora is a potent mediator of protection, probably by many complex mechanisms. We have found that colonization of germ-free mice with SFB (70 d) dramatically increases their resistance to oral listeriosis (47). The present aims are to better understand the mechanisms by which gut commensal organisms affect colonization resistance.

### SUMMARY

Microbial colonization and infection of the gut can profoundly influence the status of specific and nonspecific cellular and humoral elements of the gut mucosal immune system. These interactions contribute to the normal development of the neonatal gut mucosal immune system. Better understanding of the microbial-host gut mucosal interactions and their consequences may enable complementary or alternative approaches to prophylactic and therapeutic antibiotic therapies (48).

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