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Role of Commensal Bacteria in Development of Gut-Associated Lymphoid Tissues and Preimmune Antibody Repertoire[1]

Ki-Jong Rhee, Periannan Sethupathi, Adam Driks, Dennis K. Lanning, and Katherine L. Knight[2]

Intestinal bacteria are required for development of gut-associated lymphoid tissues (GALT), which mediate a variety of host immune functions, such as mucosal immunity and oral tolerance. In rabbits, the intestinal microflora are also required for developing the preimmune Ab repertoire by promoting somatic diversification of Ig genes in B cells that have migrated to GALT. We studied the mechanism of bacteria-induced GALT development. Bacteria were introduced into rabbits in which the appendix had been rendered germfree by microsurgery (we refer to these rabbits as germfree-appendix rabbits). We then identified specific members of the intestinal flora that promote GALT development. The combination of *Bacteroides fragilis* and *Bacillus subtilis* consistently promoted GALT development and led to development of the preimmune Ab repertoire, as shown by an increase in somatic diversification of VDJ-Cμ genes in appendix B cells. Neither species alone consistently induced GALT development, nor did *Clostridium subterminale*, *Escherichia coli*, or *Staphylococcus epidermidis*. *B. fragilis*, which by itself is immunogenic, did not promote GALT development; hence, GALT development in rabbits does not appear to be the result of an Ag-specific immune response. To identify bacterial pathways required for GALT development, we introduced *B. fragilis* along with stress-response mutants of *B. subtilis* into germfree-appendix rabbits. We identified two Spo0A-controlled stress responses, sporulation and secretion of the protein YqxM, which are required for GALT development. We conclude that specific members of the commensal, intestinal flora drive GALT development through a specific subset of stress responses. *The Journal of Immunology,* **2004, 172: 1118–1124.**

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The intestine is densely populated with bacteria, making it an important site for host-microbe interactions. Although research has focused mainly on intestinal pathogens that can cause localized and systemic infections, most intestinal microflora are not harmful, but, instead, are beneficial to the host (1). The list of beneficial functions attributed to intestinal bacteria continues to grow and includes nutrient processing (2), regulation of intestinal angiogenesis (3), development of gut-associated lymphoid tissues (GALT)[3] (4), induction of oral tolerance (5), mucosal immunity (6), and diversification of the preimmune Ab repertoire (7). It is also becoming increasingly clear that the lack of proper interactions between bacteria and the human host contributes to the prevalence of allergies and Crohn's disease in developed countries (8, 9).

In rabbits, commensal bacteria are required not only for development of GALT, as is the case in most mammals, but also for generation of a diverse preimmune Ab repertoire. Whereas the preimmune Ab repertoire in humans and mice is generated in the bone marrow through combinatorial joining of multiple V, (D), and J Ig gene segments, the repertoire in chickens and rabbits is generated by limited combinatorial joining of Ig genes, followed by somatic diversification of the resulting rearranged Ig genes in GALT. In rabbits, although >100 potentially functional V_H gene segments are available within the Ig H chain locus, the 3′-most V_H gene segment, V_H1, is used in over 80% of VDJ gene rearrangements. Newly generated B cells migrate to GALT after birth, and these cells subsequently proliferate extensively and somatically diversify their Ig genes (10, 11). We refer to the IgM^+ B cell repertoire generated at this time as the preimmune repertoire (12).

Several studies showed that intestinal flora are required for GALT development. Perey and Good (13) showed that follicular development was arrested in rabbit appendices that had been surgically ligated at birth to prevent microbial colonization. When the ligated appendix was reconnected with the intestinal lumen, follicular development was restored. Štěpánková et al. (4) found that the appendices of germfree rabbits were markedly underdeveloped and contained reduced numbers of lymphoblasts and lymphocytes. These rabbits also lacked natural antibacterial and hemolytic Abs and were either unresponsive or poorly responsive to immunization with several Ags (14). These observations suggest that intestinal microflora are essential for B cell expansion, GALT development, and generation of a normal Ab repertoire in rabbit.

In previous studies, we examined diversification of the Ab repertoire in sterilely derived rabbits whose intestinal microflora differed from that of conventionally raised rabbits and found that GALT was underdeveloped and that most VDJ-Cμ genes in the peripheral blood were undiversified. We concluded that not all commensal bacterial species promote GALT development and Ab repertoire diversification equally well (7); instead, these processes are promoted by a specific subset of species. The commensal relationship between the host and intestinal microflora has been difficult to study because of the complexity of the host-bacterial system. Not only are over 300 different bacterial species estimated to

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[3] Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; GF-Apx, germfree appendix; TRITC, tetramethylrhodamine isothiocyanate.

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reside in the intestine (any of which could influence GALT development), but their roles must be studied in the context of the host.

To identify bacteria that can induce both GALT development and a diverse preimmune Ab repertoire in rabbits, we introduced bacterial isolates from the cecum, either singly or in combination, into germfree appendices (GF-Apx) of 4-wk-old rabbits and assessed GALT development and somatic diversification of the Ig genes.

Materials and Methods

Germfree-appendix (GF-Apx) rabbits

Within 24 h after birth, the appendix lumen was flushed with 0.5 ml antibiotics (50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml metronidazole) and surgically ligated at the cecal-appendix junction to prevent colonization of the appendix by indigenous microflora, as reported previously (7). At 4 wk of age, no cultivable bacteria (aerobic or anaerobic) were present in the appendix lumen. Bacterial suspensions (1×10^9 CFU) in PBS were then injected surgically into the lumen, and 3 wk later three segments of the GF-Apx were embedded in OCT (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen.

Immunohistology

Acetone-fixed appendix cryosections (7 μm) were stained with anti-rabbit IgM mAb (clone 367) (11), followed by biotinylated goat anti-mouse Ig and streptavidin-biotin HRP complex (Vector Laboratories, Burlingame, CA). NovaRed (Vector Laboratories) was used as a colorimetric substrate. Serial sections were also stained with an Ab (clone Ki67; BD PharMingen, San Diego, CA) to a nuclear protein expressed only in proliferating cells (15), followed by alkaline phosphatase-conjugated goat anti-mouse Ig (BD PharMingen) and Vector Red (Vector Laboratories). The sections were counterstained with Gill's hematoxylin. GALT development was assessed by immunohistology with anti-Ki67 mAb and anti-IgM mAb. Cryosections with no Ki67$^+$ B cells or tiny aggregates of Ki67$^+$ B cells were considered not developed (−), and sections with medium- to large-sized Ki67$^+$ B cell follicles were considered developed (+).

Bacterial isolates from cecal contents

Rabbit cecal contents (1 g) were treated with 70% ethanol at 4°C overnight and then washed with PBS. Ethanol-treated cecal contents were cultivated under aerobic and anaerobic conditions on brain-heart infusion agar plates supplemented with 5% defibrinated rabbit blood (BHIA-BA). Colonies were isolated and identified by DNA sequencing of PCR-amplified 16S rRNA genes using the sense primer 27f (5′-CACGGATCCAGAGTTT GAT[C/T] TGGCTCAG-3′) and the antisense primer 1492r (5′-GT GAAGCTTACGG[C/T]TACCTTGTTACGACTT-3′) (16). The amplified 16S rRNA sequence was analyzed using the Ribosomal Database Project II (http://rdp.cme.msu.edu).

Bacteroides fragilis and *Clostridium subterminale* were grown on BHIA-BA plates for 24 h. *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Staphylococcus epidermidis* were grown in brain-heart infusion medium for 14 h on a shaking incubator at 37°C. The bacteria were washed three times with sterile PBS, and 1×10^9 CFU of bacterial suspensions was injected into the lumen of the GF-Apx. In some experiments, *Escherichia coli* isolated from the rabbit cecum was used. For M cell uptake experiments, *B. fragilis* and *B. subtilis* (1×10^9 CFU each) were labeled with FITC and tetramethylrhodamine isothiocyanate (TRITC), respectively, and stored at −20°C before injection (17). In other experiments, rabbits were introduced with recombinant protein A (2.5 mg) (Sigma-Aldrich, St. Louis, MO).

Western blot analysis

B. fragilis was lysed in buffer containing 50 mM EDTA, 0.1 M NaCl, 0.1 M DTT, and 0.5 mg/ml lysozyme, and lysates were electrophoresed on 15% SDS-PAGE gels. Proteins were transferred to a polyvinylidene difluoride membrane and probed with rabbit antiserum (1/1000), followed by donkey anti-rabbit Ig HRP (The Jackson Laboratory, Bar Harbor, ME) and ECL (Amersham, Arlington Heights, IL). The positive control antiserum was obtained from a normal 4-wk-old rabbit injected i.v. with *B. fragilis* (1×10^9 CFU) and bled 3 wk later.

Nucleotide sequence analysis

The VDJ-Cμ genes were amplified by RT-PCR, and nucleotide sequences from peripheral blood and appendix were analyzed, as previously described (7). The nucleotide sequences were compared with sequences of known V_H gene segments. To determine the extent of somatic diversification, we used only the VDJ genes that appeared to use V_H1 gene segments in VDJ gene rearrangements. All sequences submitted to GenBank are available under accession numbers AY359290–AY359405.

Results

GALT development in response to intestinal microflora is a complex phenomenon that requires study in the whole animal to fully elucidate the bacteria-host interaction. In this study, we introduced enteric bacterial isolates into rabbits whose appendices were rendered germfree by microsurgery, and then we analyzed GALT development. In these rabbits, the lumen of the appendix was ligated at birth to prevent bacterial colonization, and the vasculature was left intact so that lymphocyte trafficking to and from the GF-Apx was not obstructed. The appendices of GF-Apx rabbits were devoid of proliferating B cell follicles at 4 wk of age (data not shown), as evidenced by the absence of staining of cryosections with anti-IgM and anti-Ki67 mAb, which detects a nuclear protein present in proliferating cells (15). At this time, we surgically introduced bacteria into the lumen of the GF-Apx, and 3 wk later we examined GALT development by immunohistology. Introduction of normal rabbit cecal contents into a GF-Apx resulted in robust development of proliferating B cell follicles (data not shown), demonstrating that the GF-Apx rabbit model can be used to assess GALT development in response to bacteria.

Identification of bacteria that promote GALT development

Our goal was to isolate one or a small number of bacterial species that could promote GALT development. We used ethanol to kill most bacterial species in cecal contents, and 3 wk after introducing the treated cecal contents into the GF-Apx, we found Ki67$^+$ B cell follicles (Fig. 1*A*), indicative of GALT development. B cells in the Ki67$^+$ B cell follicles expressed low levels of IgM, which is characteristic of proliferating B cells (18). As a negative control, we introduced PBS and found no Ki67$^+$ B cell follicles (Fig. 1*E*). We recovered the following six bacterial species from the ethanol-treated cecal contents: *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. fragilis*, *C. subterminale*, and *S. epidermidis*, and we found that introduction of a mixture of all six bacterial species also induced GALT development (Fig. 1*B*). To determine which of these six bacterial species induced GALT development, we introduced each isolate individually or in pairs. We found that *B. subtilis* induced development occasionally, whereas individually *C. subterminale*, *S. epidermidis*, *B. fragilis*, and *E. coli* did not induce development (Table I and Fig. 1*D*). In previous studies, the presence of *B. fragilis* correlated with GALT development (7), so we tested whether the combination of *B. fragilis* with other bacteria, which by themselves did not consistently induce GALT development, would promote development. Whereas *B. fragilis* plus *C. subterminale* or *B. fragilis* plus *E. coli* rarely induced GALT development, the combinations of *B. fragilis* plus *B. subtilis* and *B. fragilis* plus *S. epidermidis* consistently induced development (Table I and Fig. 1*C*). Thus, we identified two bacterial combinations, *B. fragilis* plus *B. subtilis* and *B. fragilis* plus *S. epidermidis*, that promote GALT development in GF-Apx rabbits.

Bacterial uptake by M cells

M cells are specialized epithelial cells in GALT that transport bacteria and particulate Ags to the underlying immune cells, thereby facilitating immune responses (19). To induce GALT development, bacterial species might require the ability to be transported by M cells. We introduced FITC-conjugated *B. fragilis* into the lumen of GF-Apx and found them inside the domes, but not in the intervening villi, demonstrating that *B. fragilis* is readily taken up by M cells (Fig. 2, *A* and *B*). Therefore, the inability of *B. fragilis*

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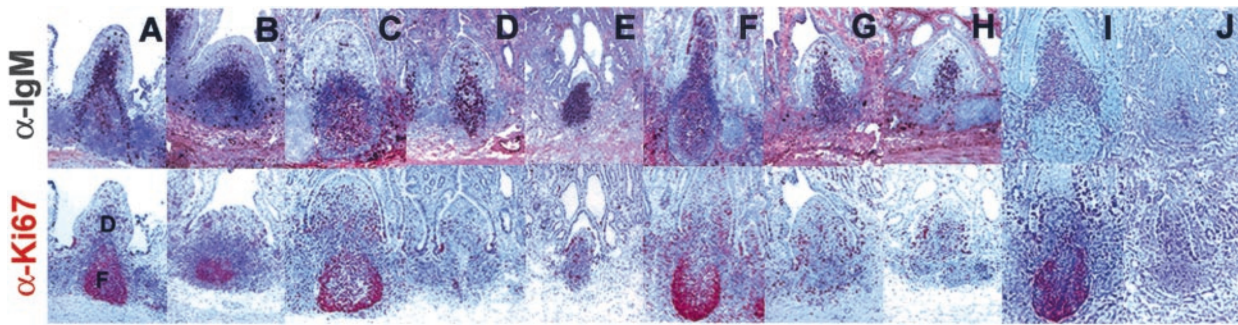

FIGURE 1. Histologic analysis of serial sections of GF-Apx after introduction of bacteria. *A*, Ethanol-treated cecal contents; *B*, *B. fragilis* plus *B. subtilis* plus *B. licheniformis* plus *B. pumilus* plus *C. subterminale* plus *S. epidermidis*; *C*, *B. fragilis* plus *B. subtilis*; *D*, *B. fragilis*; *E*, PBS; *F*, *B. fragilis* plus *B. subtilis tasA* mutant (AGS207); *G*, *B. fragilis* plus *B. subtilis spoIID* mutant (RL63); *H*, *B. fragilis* plus *B. subtilis yqxM* mutant (AGS175); *I*, *B. fragilis* plus protein A; and *J*, protein A. Sections were stained with anti-rabbit IgM mAb (*upper panels*), and serial sections were stained with anti-Ki67 mAb (*lower panels*). Sections are counterstained with Gill's hematoxylin. D, dome; F, follicle.

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alone to promote GALT development is not due to its inability to be transported across the lumen. Similarly, when we injected FITC-conjugated *B. fragilis* and TRITC-conjugated *B. subtilis* together into GF-Apx, we found both bacterial species inside the domes (Fig. 2*C*). In contrast, when we injected TRITC-conjugated *B. subtilis* alone, in two of three rabbits *B. subtilis* was not taken up by M cells effectively (Fig. 2*D*), suggesting that the presence of *B. fragilis* facilitates the uptake of *B. subtilis*. In one rabbit, *B. subtilis* alone was found inside the domes, indicating that under some circumstances, *B. subtilis* alone can be taken up by M cells.

Ag-specific response and GALT development

GALT development and the generation of a diverse Ab repertoire could result from the host's immune response to bacterial Ags. If GALT development is due to Ag-specific responses, then any bacterial species that induces such a response could be expected to induce GALT development. To test whether an Ag-specific response induced GALT development, we analyzed by Western blot the sera of GF-Apx rabbits in which *B. fragilis* had been introduced. Even though *B. fragilis* did not induce GALT development, we found that it induced a robust Ab response (Fig. 3), indicating that GALT development is not simply a consequence of an Ag-specific response to bacterial Ags.

B. subtilis *stress responses and GALT development*

Whereas *B. fragilis* alone did not induce GALT development in any of six rabbits, *B. subtilis* alone promoted GALT development in three of eight rabbits (Table I). This observation led us to hypothesize that, of the two bacteria, *B. subtilis* plays a primary role in this process. Accordingly, we sought to identify molecules or pathways in *B. subtilis* that contribute to GALT development. We introduced mutants of *B. subtilis* (Table II), in combination with wild-type *B. fragilis*, to determine which functions, when inhibited, render those bacteria unable to promote GALT development. We hypothesized that the ability of *B. subtilis* to stimulate GALT development is coupled to one or more of the known bacterial stress response pathways that would most likely become active in response to the harsh environment of the gut (20). We introduced *B. subtilis* strains bearing mutations inhibiting each of three major stress responses (all in the laboratory wild-type background PY79) (21) (Fig. 4). We first examined the effect of null mutations in the genes *sigB* and *sigD*, which control general stress responses and flagella and autolysin production, respectively (22). These strains, as well as the laboratory wild-type strain of *B. subtilis*, supported GALT development, thereby excluding *sigB* and *sigD* stress-controlled pathways as having a detectable influence on GALT development (Table II).

The *spo0A* (Fig. 4) controls a large set of postexponential phase responses in *B. subtilis*, including sporulation (23) and biofilm formation (24). To determine whether Spo0A-controlled pathways are required for GALT development, we introduced *spo0A* null mutant cells (RL891) into GF-Apx rabbits and found that they did not promote GALT development (Table II). To narrow down which of the many Spo0A-controlled responses affected GALT development, we introduced *B. subtilis* strains in which sporulation had been blocked at an early stage by a null mutation in the sporulation-specific gene, *spoIID* (RL63) (25). These bacteria did not stimulate GALT development, indicating that sporulation is required (Fig. 1*G*, Table II).

The finding that sporulation is needed for GALT development does not exclude the involvement of other Spo0A-controlled pathways. Therefore, we tested whether a recently identified Spo0A-dependent pathway, governed by the *yqxM sipW tasA* operon, is

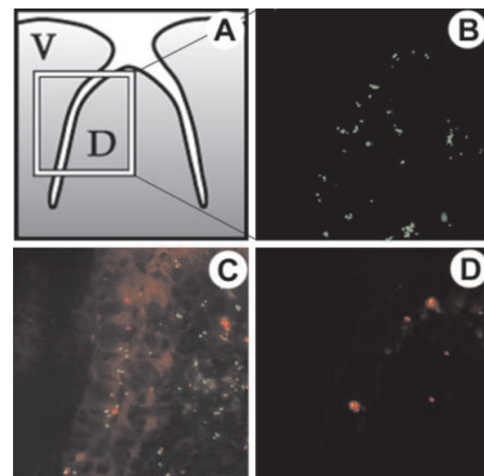

FIGURE 2. Uptake of bacteria by M cells. FITC-conjugated *B. fragilis* and/or TRITC-conjugated *B. subtilis* (1×10^9 CFU each) were introduced into GF-Apx. Three hours later, cryosections were prepared and examined by fluorescence microscopy. *A*, Schematic of appendix depicting dome (D) and intervening villous (V) regions from which micrographs were taken (boxed area). *B*, FITC-*B. fragilis*; *C*, FITC-*B. fragilis* plus TRITC-*B. subtilis*; *D*, TRITC-*B. subtilis*. Results are characteristic of three rabbits injected with *B. fragilis*; two rabbits injected with *B. fragilis* plus *B. subtilis*; and two of three rabbits injected with *B. subtilis* alone. In *C*, *B. subtilis* appears as red dots above the red fluorescence background.

Table I. *GALT development after introduction of bacteria into the GF-Apx*[a]

Inoculum[b]	Development No. Rabbits −	Development No. Rabbits +
Cecal contents		2
Cecal contents filtrate[c]	2	
Ethanol-treated cecal contents		1
Six bacteria[d]		2
B. fragilis	6	
C. subterminale	2	
S. epidermidis	2	
E. coli	2	
B. subtilis	5	3
B. fragilis + *B. subtilis*		8
B. fragilis + *S. epidermidis*		2
B. fragilis + *C. subterminale*	3	
B. fragilis + *E. coli*	3	1
Protein A	2	
Protein A + *B. fragilis*		2

[a] Bacteria (1×10^9 CFU) were introduced into GF-Apx at 4 wk of age. Three weeks later, GALT development was assessed by immunohistology with anti-Ki67 mAb and anti-IgM mAb. −, No $Ki67^+$ B cell follicles and/or tiny aggregates of $Ki67^+$ B cells; +, medium to large $Ki67^+$ B cell follicles. In each rabbit, similar results were obtained from samples taken from three different regions of the GF-Apx.
[b] The following inocula did not induce GALT development: autoclaved rabbit chow, autoclaved six bacteria (see footnote [d]), PBS, *Bacteroides uniformis*, or *E. coli* (DH10b).
[c] Cecal content filtrate was prepared by suspending cecal contents (1 g) in 1 ml of PBS and filtering through two 0.2-μm filters.
[d] Six bacteria consists of 1×10^9 CFU each of *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. fragilis*, *C. subterminale*, and *S. epidermidis*.

also required for GALT development. TasA and YqxM are secreted proteins that require the signal peptidase SipW for their secretion (Fig. 4) (26–30). We found that, whereas a *tasA* null mutant promoted GALT development (Fig. 1*F*), a *yqxM* null mutant did not (Fig. 1*H* and Table II). The mutation that inactivates *yqxM* does not prevent *sipW* expression or activity (27), which means that the inability of the *yqxM* null mutant to induce GALT development is not because of the absence of SipW. These data indicate that YqxM is required for the induction of GALT development stimulated by *B. fragilis* and *B. subtilis*.

B cell superantigen and GALT development

As described above (Fig. 3), GALT development does not appear to be driven by an Ag-specific response. Another mechanism by which GALT could develop is through Ag-independent stimulation of B cells by a molecule, such as a B cell superantigen. To test whether a B cell superantigen could induce GALT development in the GF-Apx rabbits, we introduced a model B cell superantigen, protein A of *Staphylococcus aureus* (31), into the lumen of the GF-Apx. Whereas the introduction of recombinant protein A alone did not induce GALT

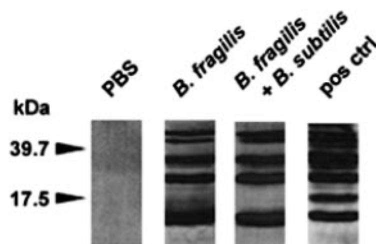

FIGURE 3. Western blot analysis of anti-*B. fragilis* Ags from sera of GF-Apx rabbits after intralumenal introduction of PBS, *B. fragilis*, or *B. fragilis* plus *B. subtilis*. Positive control (ctrl) serum is from normal rabbits injected i.v. with *B. fragilis* alone.

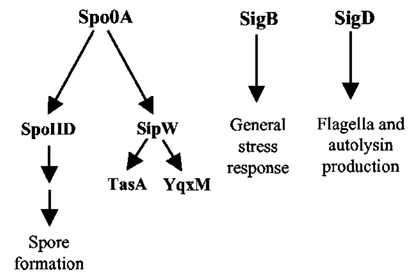

FIGURE 4. Proteins that regulate some major stress responses in *B. subtilis*.

development (Fig. 1*J* and Table I), introduction of protein A with *B. fragilis* did induce GALT development (Fig. 1*I* and Table I). These data demonstrate that the combination of a B cell superantigen with *B. fragilis* is sufficient to induce GALT development. We suggest that two signals may be required to promote GALT development, in this case, a signal through the B cell receptor and a second signal provided by *B. fragilis*.

Somatic diversification of V(D)J genes in response to B. fragilis *and* B. subtilis

To determine whether *B. fragilis* plus *B. subtilis* induce somatic diversification of Ig genes, we examined the VDJ-Cμ genes from GF-Apx rabbits injected with *B. fragilis* plus *B. subtilis* at 3 and 7 wk after injection. After 3 wk, ~50% of the V_H genes in the appendix had ≤2 nucleotide changes per V_H gene, whereas at 7 wk most (80%) had >3 nucleotide changes per V_H gene (Fig. 5). These data suggest that the Ig genes underwent somatic diversification in response to the introduction of *B. fragilis* plus *B. subtilis* in GALT.

We considered the possibility that somatic diversification of the Ig genes in the GF-Apx 7 wk after injection of bacteria was due to an infiltration of B cells with diversified VDJ genes from the periphery rather than to de novo diversification in the GF-Apx. Because Ig genes can somatically diversify in other GALT, including sacculus rotundus and the Peyer's patches, B cells in the periphery of GF-Apx rabbits are likely to be diversified. To test this possibility, we examined the VDJ-Cμ genes from the spleen of GF-Apx rabbits 3 and 7 wk after introduction of *B. fragilis* + *B. subtilis*.

Table II. *GALT development in response to* B. fragilis *and mutant* B. subtilis[a]

B. subtilis Strains	Genotype	Development No. Rabbits −	Development No. Rabbits +	Source
PY79	Wild type		3	Ref. 21
PM126	*sigB*Δ::*cat*		2	R. Losick[b] (Harvard University)
HB4035	*sigD*Δ::*kan*		2	J. Helmann[c] (Cornell University)
RL891	*spoOA*Δ::*erm*	2		R. Losick[b] (Harvard University)
RL63	*spoIID298*	3		Ref. 54
AGS175	*yqxM*Δ::*neo*[d]	2		Ref. 27
AGS207	*tasA*Δ::*spc*		2	Ref. 27

[a] All strains (congenic with PY79) were cultured 14 h in Luria-Bertani medium and washed with sterile PBS. Bacterial suspensions (1×10^9 CFU) in PBS were injected into the GF-Apx lumen and 3 wk later were examined for GALT development, as described in Table I.
[b] From whom these strains were obtained.
[c] From whom this strain was obtained.
[d] The promoter in *neo* drives expression of *sipW* and *tasA*, downstream of *yqxM*.

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We found that at both time points, the level of diversification was higher in the spleen than in the appendix (Fig. 5), indicating that most of the B cells in the GF-Apx were probably not derived from the periphery. We conclude that the Ig genes in the appendix underwent somatic diversification locally.

Discussion

The means by which commensal bacteria influence mammalian hosts is beginning to be elucidated. For example, Gordon and colleagues (3) recently showed that monoassociation of germfree mice with the intestinal commensal bacterium, *Bacteroides thetaiotaomicron*, resulted in induction of angiogenesis and production of host antimicrobial proteins (32). Using the same model, they also identified a microbial metabolic pathway that regulated production of fucosylated glycans on host enterocytes (33). Recent advances in DNA microarray analysis and identification of specific bacterial species by 16S RNA will greatly enhance the speed at which we can elucidate the molecular interplay between commensal bacteria and the host.

In this study, we used a model in which we surgically isolated the appendix, a major GALT, thereby generating a germfree compartment. The GF-Apx model has advantages over a germfree rabbit model because in germfree rabbits, the absence of intestinal microflora results not only in undeveloped GALT, but also in nutrient deprivation and bone deformities. Furthermore, germfree animals may be in an unbalanced state in which responses to stimuli are dysregulated and thus nonphysiologic. In GF-Apx rabbits, these caveats are circumvented, and the overall health and physiology of the animal are maintained, while the intestinal bacteria-host interaction can be examined.

In rabbits, intestinal flora are required not only for GALT development, but also for somatic diversification of the preimmune Ab repertoire (7). Somatic diversification of Ig genes occurs in germinal centers, sites of extensive B cell proliferation. It is generally agreed that in various species, including rabbit, proliferation of B cells is a prerequisite for GALT development and somatic diversification of Ig genes. Determining the bacterial stimuli that induce B cell proliferation in rabbit GALT will be key to understanding the process of somatic diversification in response to intestinal flora.

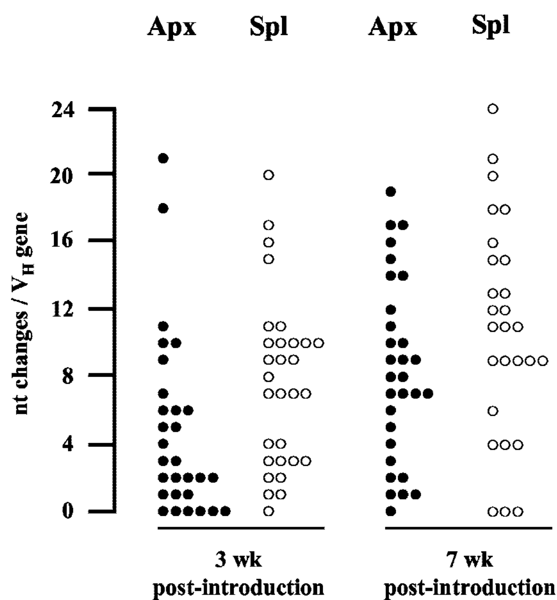

FIGURE 5. Somatic diversification of IgM VDJ genes in GF-Apx rabbits 3 and 7 wk after introduction of *B. fragilis* + *B. subtilis*. Each dot represents a sequence from the V_H region of VDJ-Cμ genes from appendices (Apx; ●) and spleen (Spl; ○) of GF-Apx rabbits. Sequences are from three GF-Apx rabbits per group.

We identified a combination of two bacteria, *B. subtilis* + *B. fragilis*, that routinely induced GALT development, as well as several bacterial isolates that either alone or in combination did not consistently induce development (e.g., *E. coli*, *C. subterminale*, *B. fragilis*, *B. fragilis* + *C. subterminale*, *B. fragilis* + *E. coli*). These data confirm and extend the findings of Lanning et al. (7) that not all bacterial species, or combinations thereof, induced GALT development. The requirement for both *B. subtilis* and *B. fragilis* to induce GALT development raises the question of whether the two species provide different, but complementing signals for development or whether one bacterial species plays the key role while the other has a passive, augmentative role. Consistent with the latter possibility, *B. subtilis* alone induced GALT development in three of eight GF-Apx rabbits, suggesting that *B. subtilis* is of primary importance and that *B. fragilis* has an augmentative role.

B. fragilis can augment *B. subtilis*-mediated induction of GALT development by enhancing uptake of *B. subtilis* by M cells. M cells are specialized epithelial cells on the follicle-associated dome epithelium in GALT (19) that transport lumenal Ags to the underlying tissues for interaction with immune cells. We found that FITC-conjugated *B. fragilis* was transported to the underlying tissues, but that this uptake was not sufficient to induce GALT development. Although we performed only a few experiments with TRITC-conjugated *B. subtilis*, it was generally transported to the underlying tissues only in the presence of *B. fragilis*. Therefore, we suggest that one role of *B. fragilis* may be to enhance uptake of *B. subtilis*. Consistent with this idea, Meynell et al. (34) observed an increase in transport of latex microspheres by rabbit Peyer's patch M cells when exposed to *Streptococcus pneumoniae*, but not to *E. coli*. We suggest that if *B. subtilis* is taken up by M cells efficiently, it alone can induce GALT development and somatic diversification of Ig genes. Furthermore, if two signals are required for initiation of these processes, *B. subtilis* alone may provide both signals. Evidence that bacterial strains can individually promote GALT development was obtained previously by monoassociation of germfree mice with *Morganella morganii* (35) or with segmented filamentous bacteria (6). Both strains induced GALT development in the absence of other bacteria.

Somatic diversification of Ig genes

We found that most VDJ-Cμ genes from appendix of GF-Apx rabbits introduced with *B. fragilis* plus *B. subtilis* were essentially undiversified (≤2 mutations) 3 wk after injection, but were diversified 7 wk after injection. Rabbit VDJ genes undergo somatic diversification via gene conversion and somatic point mutations (10, 36), and by examining the VDJ genes from GF-Apx rabbits inoculated with *B. fragilis* plus *B. subtilis*, we found several VDJ genes in which the V_H region appeared to be diversified via both somatic point mutations and gene conversion (data not shown). We suggest that the somatic diversification occurred in the GF-Apx as a result of the interaction of the bacteria with the GF-Apx. Consistent with this idea was the observation that, by Northern analysis, activation-induced cytidine deaminase, an enzyme required for somatic hypermutation (37) and gene conversion (38, 39), was expressed in the GF-Apx introduced with *B. fragilis* plus *B. subtilis* (S. L. Kalis and K. L. Knight, unpublished observations). We conclude that VDJ genes are most likely diversified in the GF-Apx in a manner similar to that of normal rabbits.

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Stress responses of B. subtilis *and development of GALT*

The finding that *yqxM* is required for GALT development clearly suggests that the protein YqxM plays a critical role in this process. The requirement for sporulation, as demonstrated by the inability of the *spoIID* mutant (which is able to secrete YqxM) (27) to promote GALT development, is more complex to interpret. The simplest explanation is that YqxM is the predominant active component required for GALT development and that sporulation is required solely to allow *B. subtilis* to survive in the gut environment long enough to promote GALT development. The other possibility is that, in addition to YqxM, a second sporulation-specific factor such as a spore coat protein is also required for GALT development.

We considered the possibility that GALT development is induced solely by the presence of viable bacteria in the GF-Apx. This, however, is not the case because GALT did not develop in response to *E. coli* or *B. fragilis* even though live bacteria were still present in the lumen (1×10^7 CFU/GF-Apx) at the end of the experiment. In fact, recovery of bacteria is not a strong predictor of GALT development, as evidenced by the absence of live *B. subtilis* from the GF-Apx after introduction of *B. fragilis* plus *B. subtilis* (data not shown).

B cell stimulation in GALT

Bacteria could stimulate B cell proliferation in GALT through a classical Ag-specific immune response, by stimulation through a B cell superantigen or by stimulation through the innate immune system. The simplest explanation is that B cells are activated by bacteria as part of an Ag-specific immune response. We do not think this is the case because *B. fragilis*, which does not induce GALT development by itself, induced a robust Ab response, comparable to that seen when animals are immunized parenterally. Sehgal et al. (40) found that the nature of somatic diversification of VDJ genes in the appendix differs from that which occurs in response to specific Ags in the spleen, further indicating that the proliferation of B cells in the GF-Apx is not due to an Ag-specific response.

If GALT development and somatic diversification of Ig genes are not driven by an Ag-specific response, then T cells might not be required for these processes. Evidence to support this idea comes from the observations that T cells are not required for germinal center formation in response to T-independent Ags (41) and that normal-sized Peyer's patches appear in T cell-deficient mice (42). Furthermore, Ig genes can undergo somatic diversification in the absence of CD40-CD40L-mediated T cell-B cell interactions in patients with X-linked hyper-IgM syndrome (43), suggesting that somatic diversification can occur in the absence of conventional B cell-T cell interactions.

Although we believe that GALT development and diversification of the Ab repertoire are not generated by an Ag-specific response, we do think that the B cell receptor is most likely required for GALT development. Transgenic chickens whose B cells expressed a truncated (V_H-less) form of surface IgM underwent rapid cell death in the bursa after hatching, suggesting that signaling through this receptor, presumably by lumenal Ags, is required for proliferation and/or survival of B cells (44).

Bacteria might promote GALT development by stimulating B cells in a polyclonal manner, via a B cell superantigen, similar to protein A of *Staphylococcus aureus* (31) and protein L of *Peptostreptococcus magnus* (45). In this scenerio, a *B. subtilis* molecule(s) could interact with the B cell receptor of all B cells at a site other than the Ag binding site, thereby directly stimulating proliferation. Such stimulation would be comparable to that of bacterial T cell superantigens that activate T cells independent of the Ag specificity of the TCR (46, 47). B cells stimulated in this manner would not be Ag specific, and further diversification would lead to an increased repertoire. The experiments with protein A demonstrated that a B cell superantigen in conjunction with *B. fragilis*, can induce GALT development. Assuming that protein A, unlike *B. subtilis*, is taken up by M cells independent of *B. fragilis*, then it appears that GALT development requires two signals, the first provided by protein A and the second provided by *B. fragilis*. Protein A is found only in *S. aureus*, which is not a significant member of the gut flora in rabbits, so if GALT development in normal rabbits is driven by a bacterial B cell superantigen, then it most likely originates from another bacterial species. In our system, we propose that such a superantigen is present in *B. subtilis* and *S. epidermidis* because both organisms, in conjunction with *B. fragilis*, can promote GALT development. In the case of *B. subtilis*, this bacterial species may contain both signals, but inefficient uptake by M cells decreases its chances of promoting GALT development alone. It is also possible that the putative B cell superantigen may not be of bacterial origin, but rather is a bacterially induced host protein or an endogenous host protein (48).

The third possibility is that bacteria stimulate GALT development through the innate immune system (49). In this case, bacterial products might react with Toll-like receptors and directly stimulate B cells to proliferate (50). Cross-linking of RP105, a Toll-like receptor family member, is known to stimulate human and murine B cell proliferation (51, 52); as such, it could promote GALT development. Alternatively, bacterial products such as LPS could stimulate macrophages and dendritic cells to secrete cytokines, which in turn could support B cell proliferation and GALT development (53).

We used a GF-Apx rabbit model to study the complex interactions between intestinal bacteria and their hosts. We isolated several bacteria naturally found in the rabbit gut environment and microsurgically manipulated rabbit appendices, and then we injected the isolates back into the ligated appendix. We found that the combination of *B. fragilis* and *B. subtilis* promoted GALT development and somatic diversification of the preimmune Ab repertoire. Furthermore, we demonstrated that two Spo0A-controlled stress responses in *B. subtilis* were required for GALT development. Future studies using this model system can elucidate, at a molecular level, the mechanisms by which intestinal bacteria stimulate host immune development.

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