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Intestinal Microflora and Diversification of the Rabbit Antibody Repertoire

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The rabbit establishes its primary Ab repertoire by somatically diversifying an initial repertoire that is limited by restricted V_{H} gene segment usage during VDJ gene rearrangement. Somatic diversification occurs in gut-associated lymphoid tissue (GALT), and by about 1–2 mo of age nearly all Ig VDJ genes are somatically diversified. In other species that are known to establish their primary Ab repertoire by somatic diversification, such as chicken, sheep, and cattle, diversification appears to be developmentally regulated: it begins before birth and occurs independent of exogenous factors. Because somatic diversification in rabbit occurs well after birth in GALT, the diversification process may not be developmentally regulated, but may require interaction with exogenous factors derived from the gut. To test this hypothesis, we examined Ab repertoire diversification in rabbits in which the appendix was ligated shortly after birth to prevent microbial colonization and all other organized GALT was surgically removed. We found that by 12 wk of age nearly 90% of the Ig VDJ genes in PBL were undiversified, indicating that intestinal microflora are required for somatically diversifying the Ab repertoire. We also examined repertoire diversification in steriley derived Remote colony rabbits that were hand raised away from contact with conventional rabbits and thereby acquired a different gut microflora. In these remote colony rabbits, GALT was underdeveloped, and 70% of the Ig VDJ genes in PBL were undiversified. We conclude that specific, currently unidentified intestinal microflora are required for Ab repertoire diversification. The Journal of Immunology, 2000, 165: 2012–2019.

The rabbit is one of a few vertebrate species known to make limited use of combinatorial joining of multiple V_{H}, D_{H}, and J_{H} gene segments during Ig heavy chain gene rearrangement. Although there are more than 100 V_{H} gene segments available within the rabbit Ig heavy chain locus, many of which appear to be potentially functional (1), the 3'-most V_{H} gene segment, V_{\text{Ig}L}, is utilized in 80–90% of VDJ gene rearrangements (2). Most of the remaining 10–20% of VDJ gene rearrangements utilize only two other V_{H} gene segments, V_{\text{Ig}x} and V_{\text{Ig}y} (3). Rabbit B cells diversify their VDJ genes in gut-associated lymphoid tissue (GALT)\(^4\) at 1–2 mo of age through two targeted mutational processes: a somatic gene conversion–like mechanism that transfers tracts of nucleotide sequence from upstream V_{H} gene segment donors into the rearranged V_{H} gene segment (4), and somatic hypermutation that distributes point mutations throughout the entire VDJ gene (5–7). At that time, the B cell population is also expanded through proliferation in GALT. B cell proliferation and Ig VDJ gene diversification in GALT results in a large population of B cells that express a wide range of Ab specificities.

Several studies have established the importance of GALT in B cell proliferation and VDJ gene diversification. Weinstein et al. (7), for example, determined VDJ gene nucleotide sequences from appendix follicles of 6-wk-old rabbits and found highly diversified, clonally related VDJ genes, which strongly implicates rabbit appendix follicular germinal centers as sites of VDJ gene diversification. In an earlier study, Cooper et al. (8) surgically removed GALT from newborn rabbits and found that they had decreased serum Ig levels, fewer circulating lymphocytes, and diminished Ab responsiveness to several Ags. More recently, Vajdy et al. (9) examined diversification of the Ab repertoire in GALT-less rabbits, similar to those used in the study by Cooper et al. (8), and found that, until 5 mo of age, IgM VDJ genes from peripheral B cells had undergone little diversification compared with those of control rabbits. In addition, the percentage of peripheral B cells was significantly reduced in the rabbits lacking GALT. These studies demonstrate that GALT is the major site of B cell proliferation and VDJ gene diversification in the young rabbit.

Several studies demonstrated that GALT development in rabbits requires gut microflora. In studies in which the appendix was surgically ligated shortly after birth, thereby preventing microbial colonization, follicular development of the appendix was arrested (10, 11). When the appendix was reconnected with the intestinal lumen within 5 mo after birth, follicular development was restored (10). Similarly, studies of germfree rabbits indicate that gut microflora is important for normal GALT development. Štěpánková and Kovář (12, 13) found that the appendices of germfree rabbits were markedly underdeveloped and contained reduced numbers of lymphoblasts and lymphocytes. Tlaskalová-Hogenová and Štěpánková (14) found that germfree rabbits lacked natural antibacterial and hemolytic Abs and were either unresponsive or poorly responsive to immunization with several Ags. These observations suggest that gut microflora is essential for the development of rabbit GALT and humoral immune responses.
In species such as chicken, sheep, and cattle that are known to develop primary Ab repertoires through somatic diversification with little contribution from combinatorial rearrangement, diversification appears to occur independent of exogenous Ag. For example, in the chicken, VDJ genes are diversified somatically in the bursa of Fabricius during embryogenesis (15–18). Similarly, in cattle, VJ genes are diversified in the fetal liver and spleen (19, 20). In sheep, most VJ genes diversify somatically in ileal Peyer’s patches before and shortly after birth (21, 22). Sheep VJ gene diversification appears to be developmentally regulated rather than driven by exogenous Ag because, even though diversification increases after birth, following microbial colonization of the gut, diversification of Ig genes can occur in sterile ileal loops (21). In contrast, diversification of the primary Ab repertoires of rabbits and probably swine (23) occurs after birth. In rabbits, repertoire diversification occurs 1–2 mo after birth in GALT that is associated with a colonized gut (24). We hypothesize that diversification of the primary Ab repertoire in rabbits requires gut microbial flora.

To test this hypothesis, we examined VDJ gene diversification in rabbits designated ligApx, in which the appendix was ligated at birth to prevent microbial colonization and all other organized GALT was surgically removed. We also examined GALT development and VDJ gene diversification in steriley derived rabbits that were maintained away from contact with the microflora of conventional rabbits.

Materials and Methods

Development of rabbits with a ligated appendix (ligApx)

Within 24 h after birth, the sacculus rotundus was removed from rabbits and the appendix was isolated from cecal contents by ligating it in two places at its junction with the cecum and severing it between the ligations. Care was taken not to alter the appendix vasculature. The pups were returned to the nesting box and were raised by the dam. The Peyer’s patches were removed at 5 wk of age when they became macroscopically visible.

Hand-raised remote colony rabbits

Rabbit pups were delivered by sterile hysterectomy or sterile cesarian section and transferred to autoclaved filter-top plastic tubes containing toweling for nesting. The filter-top tubes were placed in cages in small isolation cubicles in a temperature- and humidity-controlled room on an automatic lighting cycle. Two littermate controls were hand-raised in our conventional rabbit colony so they could acquire conventional rabbit intestinal microflora. All of the pups were hand-fed a modified VA 2-b milk diet (adapted from Ref. 25) ad libitum twice a day. The control littermates’ milk diet was intentionally contaminated one time with fecal material from a conventional rabbit colony to ensure that they were colonized by conventional microflora. At 4–5 wk of age, the pups were weaned from the milk formula diet and fed an autoclaved solid food diet (adapted from Ref. 25).

Intestinal flora from mice and conventional rabbits were introduced into remote colony rabbits as follows: −0.1 g of fecal material recovered from laboratory mice or conventional rabbits was suspended in 0.9 ml of sterile buffer (26), and the fibrous material was allowed to settle. Twenty-five microliters of the cleared suspension was diluted in 5 ml of milk formula and fed to the rabbits once during the first 2 wk of hand-rearing.

Nucleotide sequences of VDJ genes

PBL were recovered as buffy coats and lysed in guanidinium isothiocyanate. RNA was isolated either by ultracentrifugation on CsCl gradients or with TRIzol (Life Technologies, Grand Island, NY), and first-strand cDNA was synthesized using oligo(dT) as a primer (27). The VDJ-C gene sequences were PCR amplified from the cDNA by using primers specific for exon 1 of Cµ (24) and a conserved region in the Vµ leader (28). The 600-bp PCR products were gel-purified and cloned into the pGEM-T vector (Promega, Madison, WI), and the nucleotide sequences of the cloned VDJ genes were determined using an automated ABI Prism 310 sequencer with Big Dye-labeled terminators (Perkin-Elmer Applied Biosystems, Foster City, CA). The Vµ gene segments used in the VDJ genes were identified by comparing the nucleotide sequences to known germline Vµ gene segments. The germline gene with the sequence most similar to that of the VDJ gene was designated as the utilized gene and was used for determining the extent of somatic diversification of the VDJ gene. Diversification was determined by counting the number of nucleotide changes occurring in the 300-bp region between the beginning of framework 1 and the end of the Vµ gene segment. In a few cases, the number of nucleotide changes appeared to be excessively large by comparison to all known germline Vµ gene segments, suggesting that an unidentified Vµ gene segment was used in the VDJ gene. The number of nucleotide changes in the most highly mutated sequence from the control rabbits (21 nt changes) was therefore set as an upper limit, and sequences with more than 21 nt changes were excluded from our calculations. Six of the 184 total sequences were excluded on this basis. All VDJ gene nucleotide sequences were submitted to GenBank and are available under accession numbers AF264439–AF2644616.

VDJ-Cy genes were PCR amplified from PBL cDNA by using a 5’ primer from a conserved region in the Vµ leader (28) and a 3’ primer, Gammnapr (5’-GTGGAAATTCGGAGGGGCCATGGGAA-3’), specific for exon 1 of Cy. Cloning, sequencing, and determination of mutations in VDJ-Cy genes were performed as described above. Sequences of 10 VDJ-Cy genes were submitted to GenBank and are available under accession numbers AF264429–AF264438.

Analysis of gut microflora

Fresh fecal pellets were collected from each rabbit for analysis of gut flora. As described in Keshavarzian et al. (26), 0.1 g of feces was dissolved in 0.9 ml buffer, and serial 10-fold dilutions were prepared. We plated 100 ml of appropriate dilutions, in duplicate, on a series of selective media and incubated them under both aerobic and anaerobic conditions. Morphologically distinct colonies were counted and expressed as colony-forming units per gram of feces. Gram-negative aerobes and facultative anaerobes were cultivated by aerobic incubation on MacConkey agar. Gram-positive aerobes and facultative anaerobes were cultivated by incubation in candle jars on phenolethanol agar (PEA). Bacteroides spp. were cultivated by anaerobic incubation on kanamycin-vancomycin agar. Gram-positive and Gram-negative anaerobes other than Bacteroides spp. were cultivated by anaerobic incubation on PEA. In most cases, microorganisms were identified only on the basis of colony morphology and Gram stain. Microorganisms of particular interest were identified to genus and species level with the ANA-II anaerobe ID kit (Innovative Diagnostic Systems, Norcross, GA) and the BBL Crystal Enteric/Nonfermenter ID System kit (Becton Dickinson, Cockeysville, MD) or by the Loyola University clinical microbiology laboratory.

Immunohistochemical analysis of GALT thin sections and whole mounts

Tissues were harvested and frozen in OCT embedding medium (Tissue Tek, Sakura Finetech, Torrence, CA) in liquid nitrogen. Tissue sections (7 µm) were cut on a cryostat microtome, fixed in cold acetone, and blocked with goat serum. Sections were stained with the following rabbit-specific or cross-reactive primary Abs: 367, a mouse anti-rabbit µ-chain mAb for identification of B cells (9); L11/135, a mouse anti-rabbit CD43 mAb for identification of T cells (clone obtained from T. J. Kindt, National Institutes of Health, Bethesda, MD); Ki-67 mAb for identification of proliferating cells (29) (PharMingen, San Diego, CA); MECA-79, a mouse anti-rat vimentin mAb to identify M cells (32) (Sigma, St. Louis, MO); as a secondary Ab, biotinylated goat anti-mouse polyclonal Ab was used followed by ABC-AP or ABC-HRP (Vector Laboratories, Burlingame, CA), and Vector Red alkaline phosphatase substrate or VIM-13.2, a mouse anti-rat vimentin mAb to identify M cells (32) (kindly provided to us by Dr. Thomas Tedder, Duke University, Durham, NC); and VIM-13.2, a mouse anti-rat vimentin mAb to identify M cells (32) (Sigma, St. Louis, MO). As a secondary Ab, biotinylated goat anti-mouse polyclonal Ab was used followed by ABC-AP or ABC-HRP (Vector Laboratories, Burlingame, CA), and Vector Red alkaline phosphatase substrate or VIM-13.2, a mouse anti-rat vimentin mAb to identify M cells (32) (Sigma, St. Louis, MO). As a secondary Ab, biotinylated goat anti-mouse polyclonal Ab was used followed by ABC-AP or ABC-HRP (Vector Laboratories, Burlingame, CA), and Vector Red alkaline phosphatase substrate or VIM-13.2, a mouse anti-rat vimentin mAb to identify M cells (32) (Sigma, St. Louis, MO).

Quantitation of B cells and serum Ig in peripheral blood

Flow cytometry. The percentage of IgM-expressing B cells in PBL was determined by staining 1 × 10⁶ PBL, isolated as buffy coats, with a mouse anti-rabbit µ-chain mAb (367), followed by secondary staining with FITC-conjugated goat anti-mouse Ig Ab. An isotype-matched mAb was used as a negative control. Cells were counted on either FACSCount™ or FACS-Can™ (Becton Dickinson, San Jose, CA) or Beckman-Coulter EPICS-Cflow cytometers (Beckman Coulter, Fullerton, CA). ELISA. Serum IgM was quantitated by ELISA using microtiter plates coated with goat anti-rabbit µ-chain Ab followed by biotinylated mouse anti-rabbit µ-chain mAb and an avidin-biotin-HRP complex (Vectastain).
ABC Kit, Vector Laboratories) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma). Serum IgA was quantitated using microtiter plates coated with mouse anti-rabbit α-chain mAb (anti-α102) (35) and biotinylated goat anti-rabbit α-chain polyclonal Ab. IgM and IgA of known concentrations were used as standards. Color development was read at 405 nm in an ELISA plate reader. Final values represent the average of duplicate measurements.

Radial immunodiffusion. Serum IgG was quantitated by radial immunodiffusion using goat anti-Fcγ antiserum in 1% Noble agar (in 0.2 M borate saline buffer, pH 8.6), supplemented with 2% polyethylene glycol (PEG) 8000. IgG of known concentrations were used as standards. Gels were stained with GELCODE Blue Stain Reagent (Pierce, Rockford, IL). Final values represent the average of quadruplicate measurements.

Results
Ig gene diversification in ligApx rabbits

In previous studies, we showed that GALT is required for somatic diversification of Ig VDJ genes, which occurs at ~4–8 wk of age (9, 24). In the present study, we tested whether interaction between gut microflora and GALT is required for this diversification. We developed ligApx rabbits in which the sacculus rotundus and the Peyer’s patches were removed soon after birth and the appendix was ligated at birth to prevent microbial colonization, yet left the vasculature undisturbed. In four rabbits, the appendix re-established connection with the cecum and its contents, so these rabbits served as controls for the ligApx rabbits. In three of the control rabbits, the appendix reconnected with the cecum before removal of Peyer’s patches at 5 wk of age; in the fourth control, reconnection occurred between 5 and 12 wk of age. A total of seven ligApx rabbits and four controls were used in this study.

We performed laparotomies on all seven ligApx rabbits and four control rabbits at 3 mo of age and found that the appendices of the control rabbits were well developed and indistinguishable from those of conventional rabbits of similar age, but the appendices of the ligApx rabbits were undeveloped. Histologic analysis of sections of those appendices showed no evidence of follicular development (Fig. 1A). We sacrificed one of the ligApx rabbits and attempted to cultivate bacteria from the luminal contents of the appendix. We found no bacterial growth under aerobic or anaerobic conditions, and we conclude that the appendix ligations prevented microbial colonization.

To test whether VDJ-Cμ genes expressed in ligApx rabbits had undergone somatic diversification, we examined VDJ-Cμ genes cloned from cDNA prepared from PBL. At ~3 mo of age, we PCR amplified and cloned 55 VDJ-Cμ genes from ligApx rabbits and 30 VDJ-Cμ genes from control rabbits and determined their nucleotide sequences. We compared these sequences with those of the germline VH gene segments used in the VDJ gene rearrangements (Fig. 2, left). In contrast, nearly all of the nucleotide sequences (87%) from the ligApx rabbits had 0 or 1 nt change in the VH gene (Fig. 2, right). We found evidence of gene conversion, suggested by tracts of nucleotide changes and codon insertions and/or deletions, as well as of somatic hypermutation, as assessed by accumulation of point mutations in the D regions, in the diversified VDJ gene sequences of both groups of rabbits (Fig. 3). These results demonstrate that the VDJ genes in the ligApx rabbits, in which organized GALT had no interaction with microbial flora, had undergone little or no somatic diversification. We conclude that interaction between GALT and intestinal microbial flora is required for somatic diversification of the primary Ab repertoire.

Ig gene diversification in remote colony rabbits

To determine whether a particular microbial flora is required for GALT development and repertoire diversification, we derived rabbit pups sterilely and hand-reared them on a sterile diet at a remote site. Although these rabbits, designated remote colony rabbits, were raised in open cages and were not germfree, they had no contact with conventional rabbits and their microflora. As controls, littermates were hand-raised on the same sterile diet in our conventional rabbit colony so they could acquire conventional rabbit microflora.

We assessed the extent of Ab repertoire diversification in 3-mo-old remote colony rabbits and found that, with the exception of one rabbit, the VDJ gene sequences from the eight remote colony rabbits were nearly identical (0 or 1 mutation) to the germline VH genes used in the VDJ gene rearrangements (Fig. 4A, right). In contrast, 70% of the VDJ gene sequences from the two control rabbits contained two or more mutations (Fig. 4A, left). As in the ligApx rabbits and their controls, we found evidence of gene conversion and somatic hypermutation in the diversified VDJ gene sequences of both groups of rabbits. These results show that most sterilely derived rabbits that are not colonized by conventional rabbit gut microflora undergo
little diversification of their VDJ genes. One of the remote colony rabbits, however, developed normal GALT and a diversified Ab repertoire by 3 mo of age, and two others did so at 4–5 mo of age. However, most remote colony rabbits did not diversify their VDJ genes for as long as we continued to monitor them (1.5 years for two of the rabbits), showing that the Ab repertoire did not develop normally in most of the sterilely derived rabbits (7 of 10) that were not exposed to conventional rabbit flora.

Introduction of normal gut flora into remote colony rabbits

To directly test whether gut flora can drive Ab repertoire diversification, we introduced normal rabbit or mouse flora into the milk of three remote colony rabbits within the first 2 wk after birth. We examined diversification of the VDJ genes of PBL at 3 mo of age and found that the VDJ genes of all three rabbits had multiple nucleotide changes (Fig. 4B), comparable in number to those of the control rabbits with diversified repertoires (Fig. 4A, left). We conclude that, while the microbial flora of remote colony rabbits does...
not promote somatic diversification of the Ig repertoire, intestinal microflora from normally raised rabbits and mice can promote diversification.

We compared the cultivable gut microflora of remote colony rabbits with that of conventional rabbits to identify microorganisms that can potentially promote Ab repertoire diversification. We cultured dilutions of fecal pellet suspensions on a variety of selective media under aerobic and anaerobic conditions and monitored changes in the gut microflora over time. We found that the remote colony rabbits differed strikingly from rabbits raised in the conventional colony because they had undetectable numbers of non-spore-forming obligate anaerobes, primarily Bacteroides spp., which are normally a predominant group in cultivable rabbit gut microflora (36) (Fig. 5). Although these data identify Bacteroides spp. as candidate microorganisms that may promote Ab diversification, the intestinal microbial flora of the two groups of rabbits probably also differ considerably in uncultivable microorganisms, which comprise the majority of intestinal microflora.

GALT structure

We examined the appendix, sacculus rotundus, and Peyer’s patches of 10 remote colony rabbits and 2 control rabbits by laparotomy at 3–4 mo of age. Although the GALT of the control rabbits and 2 of the remote colony rabbits were indistinguishable from that of similarly aged conventional rabbits, GALT of 8 remote colony rabbits exhibited markedly underdeveloped follicular structure. The limited GALT development was readily apparent in hematoxylin and eosin-stained appendix sections (Fig. 1C). The follicles of control rabbits were similar to those of conventional rabbits (data not shown), but the follicles of the remote colony rabbit were strikingly smaller than those of either control or conventional rabbits (compare Fig. 1, B and C). Follicular expansion did not occur in most of the remote colony rabbit appendices. Further, the domes of the appendix of the remote colony rabbit were situated well beneath the villus epithelium, unlike those of control and conventional rabbits, which protruded through the villus epithelium and were in direct contact with the luminal contents. Although the appendices of most remote colony rabbits were underdeveloped, the extent of GALT development in these rabbits was significantly greater than that seen in the ligApx rabbits (compare Fig. 1 A and C). When we examined the GALT of the remote colony rabbits into which we intentionally introduced normal rabbit or mouse flora, we found that these rabbits had well-developed GALT (data not shown). These data show that GALT development and Ab diversification occurred after introduction of intestinal contents of either rabbits or mice, presumably as a result of the microbial flora.

We also examined the appendix of remote colony rabbits by immunohistochemical analysis and found that, as in conventional rabbits, the follicles of the remote colony rabbit appendix were populated by B lymphocytes and the interfollicular regions by T lymphocytes, and the domes contained a mixed population of B and T lymphocytes (Table I). Further, the distributions of L-selectin and the MECA-79 Ag were similar to those in appendices of conventional rabbits and M cells were present in the domes of both the remote colony and conventional rabbits, as detected by an anti-vimentin mAb and the lectin VVA (33). The most striking differences between the appendices of normal and remote colony rabbits was found in the levels of follicular B cell proliferation. While high levels of B cell proliferation were found throughout the entire volume of the follicles of conventional rabbits (Fig. 1D), proliferation in the remote colony rabbit appendix was largely restricted to the outer margins of the follicles (Table I; Fig. 1E). We conclude that, although the appendices of the remote colony rabbits were similar to those of conventional rabbits in basic structural organization and in distribution of B and T lymphocytes, they had much lower levels of follicular B cell proliferation.

Quantitation of B cells and serum Ig in peripheral blood of remote colony and ligApx rabbits

Because GALT of the ligApx and remote colony rabbits was underdeveloped, we tested whether B cell expansion was also impaired. We determined the percentage of B cells in PBL by immunofluorescence using flow cytometry and found that, in ligApx and remote colony rabbits, the percentage of B cells was less than that in control and conventional rabbits (Fig. 6A). To determine

| Table 1. Immunohistochemical analysis of appendix from conventional and remote colony rabbits* |
|---------------------------------|------------------|------------------|
| Reagent                         | Cells and Structures Stained | Convolutional Rabbits | Remote Colony Rabbits |
| Anti-μ                          | B cells in domes, follicles  | +++              | ++               |
| Anti-CD43                       | T cells in domes, interfollicular regions, M cell pockets | +++              | ++               |
| Ki-67 mAb                       | Proliferating cells         | +++              | +                |
| Anti-L-selectin                 | T cells in interfollicular regions, M cell pockets      | +++              | +++              |
| MECA-79 mAb                     | Follicles, lymphatics       | +++              | +++              |
| Anti-vimentin                   | M cells                     | +++              | +++              |
| VVA                            | M cells                     | +++              | +++              |

*Appendix sections were stained with various reagents, and the extent of staining was assigned a value of + for weak staining to + + + for intense staining.

**Proliferating cells were largely confined to the outer margins of the follicles of remote colony rabbit appendix, whereas they were present in large numbers throughout the follicles of conventional rabbit appendix.
whether the decrease in the percentage of B cells was accompanied by a decrease in serum Ig levels, we quantitated levels of serum IgG, IgM, and IgA. We found that serum levels of all three Ig isotypes were reduced in remote colony rabbits compared with those of conventional rabbits (Fig. 6, B–D). In ligApx rabbits, although serum levels of IgM and IgA were similar and reduced, respectively, compared with those of conventional rabbits, we found that the ligApx rabbits had serum IgG levels 3.5 times higher than control and conventional rabbits (Fig. 6 B). We found that VDJ-C\(\gamma\) genes RT-PCR-amplified from PBL of four ligApx rabbits were diversified (sequences from CDR1, CDR2 and D region of representative VDJ-C\(\gamma\) genes are shown in Fig. 7). We conclude that B cell expansion, as measured by percentage of B cells in peripheral blood, was impaired in ligApx and remote colony rabbits and, although this was reflected in generally lower serum levels of Ig isotypes, serum IgG levels in the ligApx rabbits were significantly higher than those of control and conventional rabbits. The levels of serum IgA in the remote colony rabbits and in ligApx rabbits were lower than in normal rabbits, presumably because these rabbits had little or no GALT, the tissues from which most IgA B cells develop.

Discussion

We investigated the requirement for intestinal microflora in somatic diversification of the rabbit Ab repertoire. First we examined Ig gene diversification in rabbits with all organized GALT surgically removed, except for the appendix, which was ligated at birth to prevent microbial colonization. We found that the IgM VDJ genes of these rabbits had almost no somatic diversification and that their appendices were histologically undeveloped, with no follicle formation. The lack of somatic diversification and follicular development of the appendices was not the result of surgical trauma because, in control rabbits that underwent identical surgical procedures, GALT development and Ig gene diversification were normal. Instead, our results strongly suggest that interaction between GALT and intestinal microflora is required for Ab repertoire diversification. We also examined Ig gene diversification in rabbits that were steriley derived and hand raised without contact with conventional rabbits and their microflora. These remote colony rabbits, however, were not raised under germfree conditions, and they acquired microbial flora that, although similar in numbers of cultivable bacteria, differed in composition from that of conventional rabbits. We found that most of these rabbits had underdeveloped GALT and almost no somatic diversification of their VDJ genes. These results suggest that specific species of conventional rabbit gut microflora are required for the full development of GALT, but more importantly, for diversification of the Ab repertoire.

GALT development was interrupted in the ligApx and remote colony rabbits at distinct stages. Whereas the appendices of ligApx rabbits that were not exposed to microorganisms or food Ags showed no evidence of GALT development, the GALT of remote colony rabbits, which was exposed to an unconventional flora, developed partially but lacked proliferating follicles. These results suggest that a first stage of GALT development can be stimulated simply by the presence of intestinal contents. That microbial flora is required for GALT development has been shown previously in germfree rabbits (12, 14, 37). By introducing the cecal contents of a conventional rabbit into the appendix of a ligApx rabbit, we found follicle formation within 3 wk (data not shown). These results suggest that the intestinal microflora contributes significantly to the initial development of rabbit GALT. The finding that remote

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** B cell percentages and concentration of serum Ig in ligApx and remote colony rabbits. A, B cell percentages in PBL of conventional, remote colony, ligApx, and ligApx control rabbits. Average values for each group are indicated by dashed lines. B–D, Serum IgG, IgM, and IgA concentrations, respectively, in conventional, remote colony, and ligApx rabbits. One additional ligApx control rabbit (360R1), which had high serum IgG levels (22.8 mg/ml), was not included in this figure because reconnection of the appendix occurred after laparotomy at 4–5 wk of age; in all other ligApx controls, reconnection of the appendix occurred prior to laparotomy at 4–5 wk. Average values for each group are indicated by dashed lines.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Nucleotide changes in CDR1, CDR2, and D region of IgG VDJ genes cloned from ligApx rabbits. Letters indicate nucleotide substitutions; dots indicate identity to the germline nucleotide. V\(\gamma\)1 was used in all of these clones. Three potential gene conversions are enclosed in boxes, with the nucleotide sequence of potential V\(\gamma\) gene segment donors, which are denoted in parentheses at the right of each box.
Microorganisms may drive VDJ gene diversification by directly stimulating follicular B cells to proliferate and diversify or by stimulating other cells which in turn promote B cell proliferation and VDJ gene diversification. One mechanism by which microorganisms might directly stimulate follicular B cells to proliferate is through a B cell superantigen. Although there is no direct evidence for a superantigen that stimulates rabbit B cell proliferation, certain features of rabbit B cells are consistent with the existence of such a molecule (38). For example, all rabbit V\textsubscript{H} gene segments belong to the V\textsubscript{H} III family (1), which is the major source of V\textsubscript{H} gene segments shown, in other species, to bind B cell superantigens such as Staphylococcal protein A (39) and HIV gp120 (40). Furthermore, Pospisil et al. (38) observed preferential expansion of a subpopulation of rabbit appendix B cells that express particular Ig\textsubscript{H} allotypic framework region specificities. Experiments to search for superantigens in intestinal microflora need to be performed.

Another mechanism by which B cell proliferation and VDJ gene diversification could occur in response to microbial stimulation is through Toll-like receptors that recognize highly conserved molecular patterns expressed by microorganisms (41–43). For example, RP-105, a Toll-like receptor originally described on mouse B cells and conserved in humans (44, 45), triggers an activation pathway that leads to extensive B cell proliferation and resistance against irradiation-induced apoptosis. Because Toll-like receptors can distinguish between microbial groups, for example Gram-positive and Gram-negative bacteria (46), they are potential pathways through which particular microbial species might stimulate B cell proliferation and VDJ gene diversification.

We considered the possibility that the underdeveloped GALT of the remote colony rabbits resulted from a nutritional deficit in the sterile diet rather than from the absence of specific microflora. We think this is unlikely because the GALT of control littersmates raised on the same diet developed normally. We also considered the possibility that GALT development in the remote colony rabbits was impaired by overgrowth of one or more bacteria that inhibit GALT development. Although we cannot rule out this possibility, we think it is more likely that GALT developed poorly in the remote colony rabbits because one or more specific species of the microbial flora that stimulate GALT development was absent or was present at a colonization density too low to sustain normal GALT development. Support for this idea is found in studies of germfree and gnotobiotic rodents in which different species of the intestinal microflora have been shown to directly influence various aspects of gut development, including morphology, motility, and epithelial differentiation (47–54).

Although GALT development was impaired in seven of our remote colony rabbits, three of them developed GALT indistinguishable from that of conventional rabbits, and their Ab repertoires were diversified. These rabbits were housed together with the seven rabbits in which GALT developed poorly, and from our bacterial analyses it appears that they acquired a gut microflora qualitatively similar to that of their littersmates. We suggest that the difference in GALT development between these two groups of rabbits was that the rabbits in which GALT developed normally had higher densities of one or more microorganisms capable of stimulating GALT development. However, we were unable to identify a consistent pattern of differences in bacterial colonization between these two groups of rabbits, and we suggest either that the microorganisms that drive GALT development are uncultivable under the conditions used in this study or that GALT development requires interaction among several bacterial species and that such combinations were not present in most of the remote colony rabbits.

Because follicular expansion did not occur in the GALT of either ligApx or remote colony rabbits, we predicted that expansion of the B cell population would be limited in these rabbits. Consistent with this prediction, we found that the percentages of IgM-expressing B cells in peripheral blood were significantly lower than those of conventional rabbits. A similar reduction in B cells was found in a previous study of GALT-less rabbits, from which all GALT was surgically removed (9). The reduced B cell expansion in these three groups of rabbits was reflected by lower serum concentrations of IgM and IgA. However, one surprising observation was that serum IgG levels were 3- to 4-fold higher in the ligApx rabbits. In addition, serum IgG levels were found to be increased several-fold in GALT-less rabbits (M. Vajdy and K. L. Knight, unpublished data). The elevated serum IgG levels were probably not due to inflammation resulting from surgery because, in the ligApx rabbits, no inflammation was observed during laparotomy at 5 wk of age. More importantly, the ligApx controls underwent surgical procedures identical to those of the ligApx rabbits and had normal serum IgG levels. Instead, it appears that serum IgG levels were dysregulated in the absence of developed GALT. One possible explanation for the increase is that GALT is important for the induction of tolerance to intestinal microorganisms, and in the absence of GALT, an unregulated immune response is mounted against the gut microflora. In future studies we will test this by determining the specificity of the serum IgG in these rabbits.

To understand the mechanisms by which microorganisms stimulate GALT development and Ab diversification, it will be important to identify individual bacterial species that participate in this host-microbial interaction. This can be studied by introducing bacterial isolates into the gut microflora of remote colony rabbits or into appendices of ligApx rabbits. Several candidate organisms are suggested by studies in gnotobiotic mice. For example, segmented filamentous bacteria (SFB) are uncultivable, species-specific, Gram-positive, spore-forming bacteria related to Clostridia, which upon mono-association with germfree mice induce fusocyl asialo-GMI glycolipids on small intestine epithelial cells, as well as a mucosal immune response (54). Similarly, Morganella morganii, a Gram-negative commensal bacteria found in the gut of the mouse, induces a mucosal immune response after mono-association of germfree mice (55). These bacterial species are therefore attractive candidates as microorganisms that may induce normal rabbit GALT development and somatic diversification. Because we found a striking lack of detectable colonization by Bacteroides spp. in the intestinal microflora of remote colony rabbits, these bacteria also represent potential participants in a host-microbial interaction required for rabbit GALT development. Because the results of this study indicate that normal GALT development and Ab repertoire diversification in rabbits are not dependent simply upon the presence of microorganisms in the gut, but upon interaction with specific members of normal gut microflora, understanding the nature of this host-microbial interaction will provide insight into the mechanisms underlying Ab repertoire development in rabbits.
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References