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Dependence of Antibody Somatic Diversification on Gut-Associated Lymphoid Tissue in Rabbits^{1,2}

Michael Vajdy,³ Periannan Sethupathi, and Katherine L. Knight⁴

By ~4 to 8 wk of age, the IgH VDJ genes of essentially all rabbit B lymphocytes have undergone somatic diversification. Some of this diversification occurs in the appendix, which is a gut-associated lymphoid tissue (GALT). To determine whether GALT is essential for somatic diversification, we surgically removed the appendix, sacculus rotundus, and Peyer's patches from neonatal rabbits (designated GALT-less) and examined the extent to which VDJ genes were somatically diversified. We found that the IgM VDJ genes of peripheral B cells from 2- to 5-mo-old GALT-less rabbits had undergone considerably less somatic diversification than those of control rabbits. Further, the percentage of peripheral B cells in the GALT-less rabbits was generally less than that of controls. Our data suggest that, in rabbits, the primary Ab repertoire develops in GALT, and B cell expansion also occurs there. Hence, GALT may function as a mammalian bursal homologue. *The Journal of Immunology*, 1998, 160: 2725–2729.

In mammalian species such as humans, mice, and rabbits, B lymphopoiesis occurs in bone marrow, and the B cells that undergo combinatorial joining of Ig variable (V), diversity (D), and joining (J) genes leave the bone marrow to seed the periphery (1–3). In humans and mice, combinatorial joining of multiple V, D, and J gene segments results in a vast array of V(D)J genes that form the primary Ab repertoire. This Ab repertoire is expanded further by somatic mutation after Ag stimulation (4). In chicken IgH and IgL and in sheep IgL, the primary Ab repertoire is formed by combinatorial joining of a limited number of V, D, and J gene segments followed by somatic diversification of the V(D)J genes in an exogenous Ag-independent manner (5–7). Similarly, in rabbits, the primary Ab repertoire results from combinatorial joining of primarily one V_H gene, V_HJ followed by somatic diversification by ~1 to 3 mo of age (8, 9).

Gut-associated lymphoid tissue (GALT)⁵ has a key role in somatic diversification of V(D)J genes in both chickens and sheep. In chicken, before hatching, the VDJ and DJ genes somatically diversify by gene conversion in the bursa (5, 10); in sheep, VJ genes somatically diversify in the ileal Peyer's patch (6, 7). There is evidence to suggest that in rabbits, GALT may play a similar role. Cooper et al. (11) surgically removed the appendix, sacculus rotundus, and Peyer's patches from neonatal rabbits and found that after immunization with several Ags, these rabbits responded to some of the immunogens with decreased Ab production. Further, Weinstein et al. (12) showed that VDJ genes undergo somatic diversification in the appendix of 6-wk-old rabbits, an age during

which the primary Ab repertoire is forming in rabbits (9). Together, these studies led us to investigate further whether GALT is necessary in rabbits for the somatic diversification of Ig genes that results in the establishment of the primary Ab repertoire.

To determine whether GALT is necessary for generating the primary Ab repertoire, we performed experiments similar to those of Cooper et al. (11) and surgically removed the organized GALT (i.e., appendix, sacculus rotundus and Peyer's patches) shortly after birth. Then we tested whether, in the absence of organized GALT, VDJ genes would undergo normal levels of somatic diversification within the first few weeks of life.

Materials and Methods

Removal of GALT from rabbits

The GALT of 10 rabbits was surgically altered in accordance with institutional guidelines for animal welfare using the following procedure: In nine 1-day-old rabbits, the appendix and the ileocecal junction, in which the sacculus rotundus develops, were surgically excised, and the ileum was reattached to the cecum by an end to side anastomosis. The rabbits were rested until 3 to 5 wk of age, at which time the Peyer's patches of the small intestine were surgically removed using purse-string sutures. After surgery, these rabbits were maintained under conventional conditions in our rabbit colony. We designated these rabbits GALT-less, even though they probably harbor macroscopically imperceptible lymphoid aggregates in the small and large intestines. With the exception of two rabbits that died of unknown causes at the ages of 3 and 9 mo, the GALT-less rabbits appeared healthy, with no apparent signs of infection. The growth rate of GALT-less rabbits was similar to that of control littermates (Fig. 1).

Control littermates underwent sham surgery at the same times as the GALT-less rabbits; however, on the day of birth, no tissue was removed, whereas at 3 to 5 wk of age, small pieces of small intestine were removed by purse-string suture, leaving the GALT intact. The rabbits were labeled so that each littermate had an identical letter (L or M) as well as identical numbers preceding the letter, e.g., rabbits 339M1 and 339M2 were littermates. In one rabbit, 150M1, we removed the appendix at birth as a separate test to determine whether somatic diversification occurs only in GALT. Surgery performed at 3 mo of age confirmed that the appendix had been completely excised.

Nucleotide sequence analysis

PBLs were prepared from buffy coats of whole blood as previously described (9). Erythrocytes were lysed by hypotonic shock treatment, and RNA was prepared using TRIzol according to the manufacturer's instructions (Life Technologies, Grand Island, NY). The cDNA was prepared from 3 μ g of RNA and amplified by PCR (9). The PCR products were cloned into M13 mp19, and their nucleotide sequences were determined (13, 14). The sequences were compared with those of germline V_HJ , V_HD and V_HZ as well as with the presumed germline sequence of $V_{H\kappa}$ (15); the gene that was used in the VDJ gene rearrangement was identified by its

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² All sequences reported herein have been deposited in the GenBank database and assigned accession numbers AF029916–AF029969.

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⁵ Abbreviations used in this paper: GALT, gut-associated lymphoid tissue; MLN, mesenteric lymph node; L chain, light chain; KLH, keyhole limpet hemocyanin.

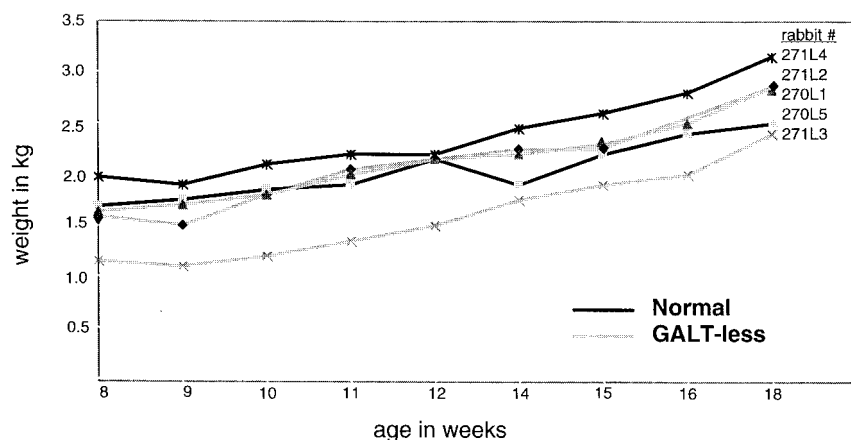


FIGURE 1. Growth curves of GALT-less and normal rabbits.

similarity to the framework regions of these germline genes. Because V_H1 is the only gene for which the nucleotide sequence is known for each of the three IgH allelic types, a^1 , a^2 , and a^3 , only the V_H1 -utilizing genes are reported in this study.

Flow cytometric analysis

PBLs, isolated from buffy coats as described above, and single cell suspensions from spleen and mesenteric lymph nodes (MLNs) were stained for light chain (L chain) expression with biotinylated goat anti-rabbit L chain and avidin phycoerythrin or stained for IgM expression with mouse anti-rabbit μ -chain mAb and FITC-goat anti-mouse Ig. The goat anti-rabbit L chain Ab was generated by immunizing a goat with purified secretory IgA and then affinity purifying the anti-L chain Ab on a rabbit IgG immunosorbant. The anti-rabbit μ mAb was generated by injecting a mouse with purified rabbit IgM (Accurate Chemical and Scientific Corp., Westbury, NY) and producing murine hybridomas by fusing spleen cells of the immunized mouse with the Ag8.653 fusion partner as described previously (16). Specificity of the anti-rabbit μ -chain mAb was established in an ELISA by showing that it did not react with purified IgG or IgA. Goat anti-mouse Ig was prepared by immunizing a goat with purified mouse IgG and then affinity purifying the specific Ab on a mouse IgG immunosorbant. Using immunofluorescence, we observed that this Ab did not react with rabbit B cells. According to ELISA, this Ab also did not react with purified rabbit IgG or IgA. The fluorescent-labeled cells were analyzed by flow cytometry (FACStar; Becton Dickinson, Mountain View, CA). The data were analyzed statistically using a Mann-Whitney U test (nonparametric).

Results

Somatic diversification of VDJ genes in rabbits with surgically altered GALT

Because Weinstein et al. (12) demonstrated that VDJ genes undergo somatic diversification in germinal centers of the appendix of 6-wk-old rabbits, we determined whether the appendix is the only immunologic site in which somatic diversification occurs. We appendectomized a rabbit on the day of birth and determined whether the VDJ genes diversified within the same time span as in normal rabbits. The IgM VDJ- $C\mu$ genes from PBLs were amplified by RT-PCR, cloned, and the nucleotide sequences were determined. We compared the nucleotide sequences of the VDJ- $C\mu$ genes with those of germline V_H1 to identify the extent to which the VDJ genes were diversified. Because Crane et al. (9) reported that the V_H genes of PBLs of normal rabbits are extensively diversified by 8 wk of age, we examined the level of somatic diversity of IgM heavy chain VDJ genes in PBLs of the appendectomized rabbit at 9 wk of age. We found that the IgM VDJ genes were diversified (Fig. 2A) with an average of 9.1 nucleotide changes per V_H region. This led us to conclude that somatic diversification of VDJ genes during the first few weeks of life can occur outside the appendix.

To investigate whether the appendix, sacculus rotundus, and Peyer's patches taken together are required for somatic diversification of VDJ genes early in life, we surgically removed all visible GALT; the appendix and sacculus rotundus were removed at birth, and the Peyer's patches were removed at ~ 4 wk of age. When these GALT-less rabbits were 10 to 12 wk of age, we then analyzed the level of somatic diversification of the VDJ genes. We amplified the IgM heavy chain VDJ genes of PBLs by RT-PCR. By nucleotide sequence analysis, we found that the V_H regions of the VDJ genes of each of three GALT-less rabbits had undergone little somatic diversification. The nucleotide sequences differed from their corresponding germline sequences by an average of 2.8 nucleotides per V_H gene (Fig. 2B). These data sharply contrasted with the data obtained from the control littermate rabbits, in which peripheral B cell VDJ genes underwent extensive somatic diversification with an average of 24 nucleotide changes per V_H region (Fig. 2C). We conclude that GALT is necessary for the somatic diversification of VDJ genes that occurs shortly after birth.

Each of the rabbits used in the study was examined surgically for the visible presence of residual remnants of GALT. We found no remnants of appendix in the appendectomized rabbit, and we found no visible evidence of residual appendix, sacculus rotundus, or Peyer's patches in all but two of the GALT-less rabbits. One of these rabbits, 170L2, had remnant appendix (approximately one-half normal size); the sacculus rotundus and Peyer's patches had been completely removed. We PCR-amplified VDJ- $C\mu$ genes from the PBLs of this rabbit at 10 wk of age, and we found an average of 10 nucleotide changes per V_H region by nucleotide sequence analysis (Fig. 2D). We conclude that VDJ genes can diversify in the appendix in the absence of both the sacculus rotundus and Peyer's patches.

The analyses described above focused on the V_H region of the VDJ genes. Because the D regions (complementarity-determining region 3) also diversify extensively within a few weeks after birth (17), we analyzed the D regions of the VDJ genes cloned from each of the rabbits used in this study. The results from the D regions were similar to those of the V_H regions. That is, in the GALT-less rabbits we found only a few mutations in the D regions (Fig. 3), whereas the D regions of VDJ genes from 10-wk-old normal rabbits were so highly diversified that it was difficult to identify the germline D gene segments used in the VDJ gene rearrangements (data submitted to GenBank). We conclude that the limited diversification of V_H genes in GALT-less rabbits extends through the entire VDJ gene.

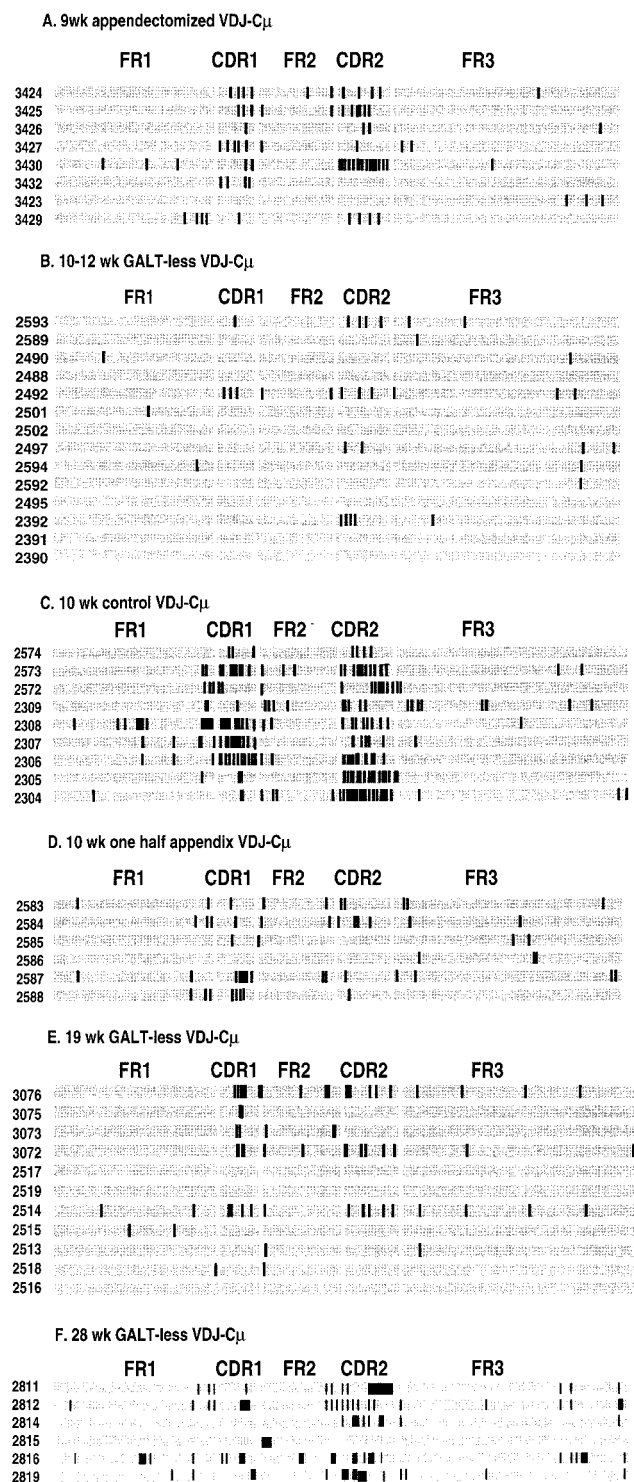


FIGURE 2. Somatic diversity of V_H regions of VDJ-C μ genes from PBLs of both rabbits with surgically altered GALT and control rabbits. Each nucleotide change, as compared with the sequence of $V_H I$ from the corresponding haplotype, is indicated by a vertical bar. *A*, A 9-wk-old appendectomized rabbit, 150M1 (V_H allotype a2,a3). Clones 3423 and 3429 appeared to utilize $V_H I$ from the a^2 allele. Clones 3424–3427, 3430, and 3432 appeared to utilize $V_H I$ from the a^3 allele. *B*, ten- and twelve-wk-old GALT-less rabbits. Clones 2589 and 2592–2594 were from rabbit 170L4 (V_H allotype a1,a2). Clones 2589 and 2593 appeared to utilize $V_H I$ from the a^1 allele; clones 2592 and 2594 appeared to use $V_H I$ from the a^2 allele. Clones 2501 and 2502 were from rabbit 271L3 (V_H allotype a1). Clones 2390, 2391, 2392, 2495, 2490, 2488, 2492, and 2497 were from rabbit 271L2 (V_H allotype a1). *C*, ten-wk-old control rabbits (V_H allotype a1). Clones 2304–2306 were from rabbit 270L5, clones 2307–2309 were

To determine whether the limited Ab diversity observed in the GALT-less rabbits would persist throughout life, we monitored the level of Ab diversity of the VDJ genes from 19- and 28-wk-old rabbits. The limited diversity of VDJ-C μ genes observed in the 10- to 12-wk-old GALT-less rabbits was also observed at 19 wk of age, at which time the V_H genes still had an average of only 5 nucleotide changes (Fig. 2*E*). Moreover, the D regions of most of these VDJ genes were relatively undiversified, and we could readily identify the germline D genes used in the VDJ gene rearrangements. By 28 wk of age, however, we found that the IgM VDJ genes of peripheral B cells in GALT-less rabbits were extensively diversified, with an average of 17.6 nucleotide changes per V_H region (Fig. 2*F*). We conclude that, while removal of organized GALT in neonatal rabbits results in decreased levels of somatic diversification of VDJ genes until early adulthood, the VDJ genes can undergo somatic diversification later in life.

B cell expansion in GALT-less rabbits

The chicken bursa and the sheep ileal Peyer's patch not only function as sites for generating Ab diversity, but also as primary lymphoid tissues for the expansion of B cells. Removal of the bursa and the ileal Peyer's patch at birth results in dramatically fewer peripheral B cells in chicken and sheep, respectively (18–21). To investigate whether rabbit GALT functions as a primary lymphoid tissue for B cell expansion, we used immunofluorescence to determine the level of B lymphocytes in the periphery in a total of seven normal and seven GALT-less rabbits. We found that at 12 wk of age the percentage of B cells in PBLs, MLNs, and spleens of GALT-less rabbits was generally <50% of that seen in littermate and age-matched normal rabbits (Fig. 4). The difference in the average percentage of B cells in the two groups is statistically significant for MLNs and spleen ($p = 0.05$). By analyzing PBLs from two GALT-less rabbits at 28 wk of age and one GALT-less rabbit at 16 mo of age, we again found a decreased percentage of B cells in PBLs. Consistent with the decreased percentage of B lymphocytes, we found nearly 50% fewer lymphocytes in GALT-less rabbits as compared with control littermates when differential counts of blood smears were used (Table I). These data indicate that the total number of B lymphocytes in the periphery of GALT-less rabbits is less than that of normal rabbits. Hence, we conclude that early in life the rabbit GALT functions as a primary lymphoid tissue for expansion of B cells.

Discussion

GALT is well known as a site of development of the primary Ab repertoire in chicken and sheep, in which extensive somatic diversification occurs in the bursa and ileal Peyer's patch, respectively (5, 6). Most of this somatic diversification occurs in chicken and sheep before and shortly after hatching and birth, respectively. In rabbits, the VDJ genes of essentially all B cells undergo somatic diversification between 4 and 8 wk of age, which generates what we define as the primary Ab repertoire (9). Weinstein et al. (12)

from rabbit 271L1, and clones 2572–2574 were from rabbit 170L1. *D*, A 10-wk-old rabbit, 170L2 (V_H allotype a1), with approximately one-half an appendix but no visible sacculus rotundus or Peyer's patches. *E*, nineteen-wk-old GALT-less rabbits (V_H allotype a1). Clones 2513–17, 3075, and 3076 were from rabbit 271L2; clones 2518, 2519, 3072, and 3073 were from rabbit 271L3. *F*, twenty-eight-wk-old GALT-less rabbits (V_H allotype a1). Clones 2811–2816 were from rabbit 271L2; clone 2819 was from rabbit 271L3. Nucleotide sequences of all clones depicted in *A* through *F* are available from GenBank under accession numbers AF029916–AF029969.

FIGURE 3. Comparison of nucleotide sequences of D regions from VDJ genes from 10- to 12-wk-old GALT-less rabbits with the nucleotide sequence of germline D regions (15). The germline Df gene segment has not been cloned (15). Consequently, N regions of the Df-utilizing clones 2490, 2495, and 2593 are approximated by comparison of cDNA sequences; the D region of clone 2492 is unknown, and the N segments are shown as part of the D region. The sequences of these clones are available from GenBank under accession numbers AF029956-AF029969.

VH-FR3	N	D	N	JH
2593 TGTGCCAGA	GGG	GGTTATACTACTG	TTAG	CATCTGGGGCCCCA Df JH4
2589 TGTGCCAGA	TTCC	ACGATGACTATG		ACTTTAAACATCTGGGGCCCCA D1 JH4
2490 TGTGCCAGA	GGG	TCCTGGTTA	CA	TAACATCTGGGGCCCCA Df JH4
2488 TGTGCCAGA	TT	ATATGCTAGTAGTAGTGGTTATTAT	T	ATGCTTTTGTGCCCTGGGGCCCCA D3 JH2
2492 TGTGCCAGA		GGGTCTCTGCATATATAGTGGTACAC		ACATCTGGGGCCCCA D? JH4
2501 TGTGCCAGA	TCCTTTTT	TAGCTACGATGACTATGGTGA	GCAGA	ACTACTTTAAACATCTGGGGCCCCA D1 JH4
2502 TGTGCCAGA	TTG	AGCTACGATGACTATGGTAT	CCC	GATGCTTTTGTGCCCTGGGGCCCCA D1 JH2
2497 TGTGCCAGA	AGTATT	TATAGCAGTGG		TAACATCTGGGGCCCCA D4 JH4
2594 TGTGCCAGA	GATTC	GCTGGTTATGCTGGTTATGGTT	CCC	TTAACTTGTGGGGCCCCA D2BJH4
2592 TGTGCCAGA	GGTGTCTTA	TAGTACGATGACTATGG	CCCCT	ACTACTTTAAACATCTGGGGCCCCA D1 JH4
2495 TGTGCCAGA	GGCCC	TCCTGGTTATAGTACTG	GTAATGGCT	ACTACTTTAAACATCTGGGGCCCCA Df JH4
2392 TGTGCCAGA	GATCCC	GGTTATTAT	GCAA	TGTGGGGCCCCA D3 JH4
2391 TGTGCCAGA	TATG	ACGATGACTAT	AG	CATCTGGGGCCCCA D1 JH4
2390 TGTGCCAGA	G	GCTACGATGACTATGGTATTAC	GGGG	GTGATGCTTTTGTGCCCTGGGGCCCCA D1 JH2

showed that some of this somatic diversification occurs in the appendix. However, that study did not determine whether the appendix was necessary for this somatic diversification. We found that when the appendix alone was removed, the VDJ genes underwent somatic diversification, albeit at a somewhat lower level than the VDJ genes of normal rabbits. Therefore, we tested the necessity of GALT for somatic diversification by analyzing the extent to which VDJ genes are somatically diversified after surgical removal of the appendix and sacculus rotundus on the day of birth and of the Peyer's patches as soon as they became macroscopically visible, at ~4 wk of age. If GALT is necessary for development of the primary Ab repertoire, we expected the VDJ genes in B cells of these GALT-less rabbits to undergo little or no somatic diversification. We found that for the first 19 wk of life, long after the time that VDJ genes undergo somatic diversification in normal rabbits, the VDJ genes in the GALT-less rabbits exhibited little or no somatic diversification. At 10 to 12 wk of age, the number of mutations per V_H gene averaged 2.8 for GALT-less rabbits compared with 24 mutations for 10-wk-old littermate control rabbits that had undergone surgery to remove a small piece of the intestine. We conclude

that organized GALT is necessary for development of the primary Ab repertoire early in life.

Although we found dramatically reduced levels of Ab diversity in the GALT-less rabbits until they reached 19 wk of age, the level of Ab diversity by 28 wk of age was similar to that of normal rabbits. Where did this diversification occur? We suggest that some of the diversification occurred in B cells located in residual, macroscopically imperceptible, lymphoid aggregates in the gut that were not removed by surgery. Presumably, some of these cells expand, undergo somatic diversification of the VDJ genes, and exit to the periphery. However, the number of B cells in these aggregates is presumably small and would not account for the large pool of B cells in the periphery. It may be that the extensive somatic diversification of VDJ genes by 28 wk of age occurs in peripheral lymphoid tissues in response to antigenic stimulation as discussed below. However, because we found that the number of peripheral B cells in GALT-less rabbits was reduced, we cannot rule out the possibility that the limited diversity of VDJ genes until 19 wk of

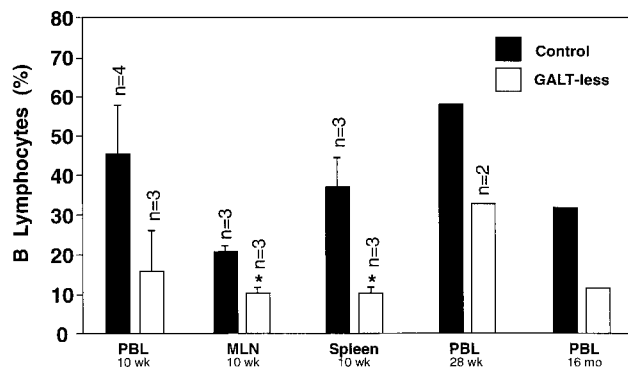


FIGURE 4. Percentage of B lymphocytes in PBLs, MLNs, and spleens of GALT-less and control rabbits. Single-cell suspensions were stained with biotinylated goat anti-rabbit L chain, avidin phycoerythrin or mouse anti-rabbit μ -chain mAb, and FITC-goat anti-mouse Ig. The control rabbits were age-matched and were as described in *Materials and Methods*. The 16-mo-old GALT-less rabbit had two macroscopic Peyer's patches. Data are presented as the percentage of B cells of total lymphocytes. Rabbits used for the 10-wk analyses were as follows: GALT-less = 339M1 (PBL, MLN, and spleen), 309M1 (PBL, MLN, and spleen), 293M3 (MLN and spleen), 170L4 (PBL); Control = 339M2 (PBL, MLN, and spleen), 303M1 (PBL, MLN, and spleen), 293M6 (MLN and spleen), 170L3 (PBL), 170L1 (PBL). Rabbits used for PBLs at 28 wk were as follows: GALT-less = 271L2 and 271L3; Control = 270L5. Rabbits used for PBLs at 6 mo were as follows: GALT-less = 270L1; Control = 270L5. The bars represent mean \pm SEM. * indicates that the percentage of B cells was statistically less than that seen in control rabbits ($p = 0.05$).

Table I. Percentage of lymphocytes in differential counts of PBL from GALT-less and control rabbits^a

Rabbit No.	Lymphocytes (%)
12-wk-old GALT-less	
170L4	49
339M1	23
309M1	6
Average	26
Control	
170L1	49
170L3	74
339M2	52
307M1	45
Average	55
28-week-old GALT-less	
271L2	12
271L3	21
270L1 ^b	25
Average	19
Control	
271L4	40
270L5	25
Average	32

^a The percentage of lymphocytes was determined from blood smears. Control rabbits, except 307M1 and 271L4 which did not undergo surgery, were as described in *Materials and Methods*.

^b Rabbit 270L1 had two macroscopic Peyer's patches.

age is due simply to the delayed development, following surgery, of mature Ig-producing cells that diversified normally.

We propose that, in normal rabbits, B cells migrate from bone marrow to the gut, where they undergo expansion and somatic diversification (9). In the GALT-less rabbits, we suggest that B cells migrate instead to the secondary lymphoid tissues, including the spleen and regional lymph nodes; in these tissues, the B cells can be stimulated by Ag to form germinal centers and undergo Ag-driven somatic diversification. Several studies support this possibility. First, in the earlier experiments of Cooper et al. (11), who surgically removed the appendix, sacculus rotundus, and Peyer's patches from young rabbits, germinal centers were found in the peripheral lymph nodes of immunized rabbits. They also found that these rabbits responded to several Ags, albeit at lower levels than normal rabbits. We immunized four of our GALT-less rabbits at 10 to 12 wk of age and found that each of them developed Ab to keyhole limpet hemocyanin (KLH) after both primary and secondary immunizations with KLH (our unpublished observations). Further, we found, in collaboration with William J. Simmons and Dr. Jeanette Thorbecke (New York University Medical Center, New York, NY), that the spleens of these immunized GALT-less rabbits had germinal centers that were equal in number and size to spleens from normal KLH-immunized rabbits (our unpublished observations). Because germinal centers are the sites at which VDJ genes undergo somatic diversification (22), we consider it likely that the VDJ genes in the germinal centers of GALT-less rabbits undergo somatic diversification. Hence, we suggest that much of the diversification of VDJ genes from the GALT-less rabbits at 28 wk of age occurred in the spleen and/or peripheral lymph nodes in response to antigenic stimulation. This idea needs to be tested directly.

The somatic diversity observed in 19- and 28-wk-old GALT-less rabbits offered the opportunity to determine whether IgH genes can undergo gene conversion outside GALT. We found many codon insertions and/or deletions in the diversified sequences that are characteristic of gene conversion events. We were unable, however, to identify potential V_H gene donors for these codons, probably because the rabbits used in this experiment had the a^1 haplotype and because only three potential donor V_H genes 5' of the utilized gene, V_{HI} , are cloned. Without identifying V_H genes that could have served as donors for the diversified regions, we are reluctant to conclude that these genes diversified by gene conversion rather than by somatic hypermutation.

Separate observations indicate that the primary Ab repertoire can be generated if only a portion of GALT exists. The VDJ genes of the rabbit with both an appendix that was one half the normal size and no sacculus rotundus or visible Peyer's patches were extensively diversified. Also, in the appendectomized rabbit with an intact sacculus rotundus and Peyer's patches, the VDJ genes were extensively diversified. Although we did not directly test whether the sacculus rotundus and Peyer's patches are able to individually promote somatic diversification, it seems likely that they can.

We found long-term deficits in the number of peripheral B cells in GALT-less rabbits. Cooper et al. (11) found lower than normal numbers of peripheral lymphocytes in rabbits with surgically removed GALT, although they did not show that the decrease was in the number of B lymphocytes. Similarly, there were fewer peripheral B cells in sheep with the ileal Peyer's patch removed early in ontogeny (21). The mechanism for maintaining a normal level of B cells in mammals is not known. However, we suggest that GALT is required to develop and/or maintain a normal number of B cells in the periphery. If GALT is a site that serves as a reservoir of B cells that undergo extensive proliferation and migrate to peripheral tissues then, in the absence of GALT, we would expect fewer B cells in the periphery. We conclude that the long-term deficit in the number of peripheral B cells in the GALT-less rabbits

results directly from the absence of GALT and, consequently, that GALT is required for the development of normal B cell numbers in the periphery.

The data from this study support the idea that in rabbits, GALT functions as a bursal homologue. Not only is GALT the site in which VDJ genes undergo somatic diversification resulting in the formation of the primary Ab repertoire, but it is also important for maintaining normal numbers of B cells. We suggest that these processes are stimulated by luminal Ags, such as viral or bacterial superantigens, that are taken up by M cells lining the follicle-associated epithelium in rabbit GALT. However, it is also possible that the expansion and somatic diversification may be a developmentally regulated process that occurs in the absence of exogenous luminal Ags. Experiments to address the potential role of luminal Ags in the expansion of B cells and diversification of Ig genes need to be performed.

Acknowledgments

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References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature* 302:575.
2. Alt, F. W., T. K. Blackwell, and G. D. Yancopoulos. 1987. Development of the primary antibody repertoire. *Science* 238:1079.
3. Nunez, C., N. Nishimoto, G. L. Gartland, L. G. Billips, P. D. Burrows, H. Kubagawa, and M. D. Cooper. 1996. B cells are generated through life in humans. *J. Immunol.* 156:866.
4. Griffiths, G. M., C. Berek, M. Kaartinen, and C. Milstein. 1984. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature* 312:271.
5. Reynaud, C.-A., V. Anquez, H. Grimal, and J.-C. Weill. 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell* 48:379.
6. Reynaud, C.-A., C. R. Mackay, R. G. Muller, and J.-C. Weill. 1991. Somatic generation of diversity in a mammalian primary lymphoid organ: the sheep ileal Peyer's patches. *Cell* 64:995.
7. Reynaud, C.-A., C. Garcia, W. R. Hein, and J.-C. Weill. 1995. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. *Cell* 80:115.
8. Knight, K. L., and R. S. Becker. 1990. Molecular basis of the allelic inheritance of rabbit immunoglobulin V_H allotypes: implications for the generation of antibody diversity. *Cell* 60:963.
9. Crane, M. A., M. Kingzette, and K. L. Knight. 1996. Evidence for limited B-lymphopoiesis in adult rabbits. *J. Exp. Med.* 183:2119.
10. Reynaud, C.-A., A. Dahan, V. Anquez, and J.-C. Weill. 1989. Somatic hyperconversion diversifies the single V_H gene of the chicken with a high incidence in the D region. *Cell* 59:171.
11. Cooper, M. D., D. Y. Perey, A. E. Gabrielsen, E. E. R. Sutherland, M. F. McKeenally, and R. A. Good. 1968. Production of an antibody deficiency syndrome in rabbits by neonatal removal of organized intestinal lymphoid tissues. *Int. Arch. Allergy Appl. Immunol.* 33:65.
12. Weinstein, P. D., A. O. Anderson, and R. G. Mage. 1994. Rabbit IgH sequences in appendix germinal centers: V_H diversification by gene conversion-like and hypermutation mechanisms. *Immunity* 1:647.
13. Yanisch, P. C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. *Gene* 33:103.
14. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463.
15. Friedman, M. L., C. Tunyaplin, S. K. Zhai, and K. L. Knight. 1994. Neonatal V_H , D, and J_H gene usage in rabbit B lineage cells. *J. Immunol.* 152:632.
16. McNicholas, J. M., M. Raffeld, M. R. Loken, H. Reiter, and K. L. Knight. 1981. Monoclonal antibodies to rabbit lymphoid cells: preparation and characterization of a T-cell-specific antibody. *Mol. Immunol.* 18:815.
17. Short, J. A., P. Sethupathi, S. K. Zhai, and K. L. Knight. 1991. VDJ genes in $V_{H\alpha 2}$ allotype-suppressed rabbits: limited germline V_H gene usage and accumulation of somatic mutations in D regions. *J. Immunol.* 147:4014.
18. Reynolds, J. D., and B. Morris. 1983. The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur. J. Immunol.* 13:627.
19. Cooper, M. D., W. A. Cain, P. J. Van Alten, and R. A. Good. 1969. Development and function of the immunoglobulin producing system: effect of bursectomy at different stages of development on germinal centers, plasma cells, immunoglobulins and antibody production. *Int. Arch. Allergy Appl. Immunol.* 35:242.
20. Kincade, P. W., K. S. Self, and M. D. Cooper. 1973. Survival and function of bursa-derived cells in bursectomized chickens. *Cell. Immunol.* 8:93.
21. Gerber, H. A., W. Trevella, and B. Morris. 1986. The role of gut associated lymphoid tissues in the generation of immunoglobulin-bearing lymphocytes in sheep. *Aust. J. Exp. Biol. Med. Sci.* 64:201.
22. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. *Nature* 354:389.