



A morphological and immunohistological study of the human and rabbit appendix for comparison with the avian bursa

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Abstract

Diversification of the primary antibody repertoire occurs in young rabbit appendix. As a prelude to molecular investigation of whether human appendix has a similar role, we compared the lymphoid morphology and distribution of common B- and T-cell subsets in frozen and/or paraffin-embedded normal appendix specimens at various ages. IgA, IgM and IgG staining patterns were similar in frozen human and rabbit appendices. The elongated follicles of the young human and rabbit appendices regressed with age to resemble Peyer's patches. Although similar in morphology to the bursa, human and rabbit appendix follicles differ in that they do not involute completely with age and contain significant numbers of germinal center (GC) T cells although the number is low early in life. If the human appendix functions as a primary lymphoid organ, it may occur during the first few months of age when the GC T-cell density is low. Published by Elsevier Science Ltd.

Keywords: Human appendix; Rabbit appendix; Lymphoid development; Chicken bursa; Germinal center; Immunohistochemistry; T-cell; B-cell repertoire

1. Introduction

The immunological development of human gut-associated lymphoid tissues (GALT) has received little study compared to animal models. The appendix is an ideal tissue for such investigation since it is commonly removed surgically. Previous studies of the rabbit appendix indicate

Abbreviations: CNMC, Children's National Medical Center; GALT, gut-associated lymphoid tissue; FR, follicular region; DZ, dark zone; GC, germinal center; JPP, jejunal Peyer's patch; LZ, light zone; SIPP, sheep ileal Peyer's patch.

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that it is an important site for development of the primary (pre-immune) antibody repertoire (reviewed in Ref. [1]). It is not known whether the human appendix plays such a role. In the young rabbit, diversification of rearranged VDJ sequences occurs in developing germinal centers (GCs) of the appendix [2] by gene-conversion-like and hypermutation mechanisms [2,3]. This role is similar to that of the sheep ileal Peyer's patches (SIPP) [4] and the avian bursa of Fabricius [5]. Thus in some respects, the rabbit appendix and SIPP may be viewed as mammalian bursal equivalents [1,2,6,7]. Common structural characteristics of some primary lymphoid organs such as the avian bursa and SIPP include relatively few or no T cells within GCs, elongated follicles, and involution of follicles before adulthood [8–12]. Although the rabbit appendix does not involute, it changes in appearance, possibly in function, and partially atrophies with age [13].

The rabbit appendix makes an important contribution to the antibody-forming potential of GALT. Neonatal appendectomy followed by early removal of other GALT reduces serum IgM and IgG levels, antigen-specific responses, and causes a temporary reduction in circulating lymphocytes [7,14,15]. Moreover, neonatal appendectomy reduces intestinal IgA, IgM and IgG levels; nearly ablates intestinal antigen-specific responses; and reduces IgA⁺ plasma cells in the intestinal lamina propria [16].

Both the human and rabbit appendix contain fewer T cells than B cells. Reported percentages of T-lymphocytes in the rabbit appendix range from 7 to 40% [17,18] and in human from 19 to 50% [19–22] which may vary according to the ages examined. The majority of T cells lie in the T-cell rich interfollicular region. A few T cells are also found in the B-cell rich follicular region (FR). T helper (CD4⁺) cells play a vital role in GC formation by providing the necessary costimulatory signal for B-cell clonal selection and expansion (reviewed in Ref. [23]). In addition, some CD8⁺T cells which secrete IL-4 can enhance B-cell proliferation [24]

Both human and rabbit appendices contain a high proportion of IgA⁺ plasma cell precursors [25,26]. In the lamina propria of rabbit GALT,

at least 80% of all Ig-containing cells are IgA⁺ plasma cells [27]. Similarly, in human GALT, 75–90% of lamina propria plasma cells are IgA⁺ (reviewed in Ref. [28]).

The goal of the present study was to compare the morphological and immunohistological changes during development of the human appendix with those of the rabbit. This comparison was needed to determine at what age(s) to conduct a molecular investigation of whether the human appendix functions in diversifying the primary antibody repertoire as occurs in the rabbit appendix and chicken bursa. We studied normal human and rabbit appendix specimens representing a wide spectrum of ages. We compared the percentage of lymphoid tissue, characterized the distributions of common B-cell subsets [IgM⁺, IgA⁺, IgG⁺, IgD⁺ (IgD is absent in rabbit)], T-cell subsets (CD4⁺, CD8⁺), and nuclear proliferation antigen positive (Ki-67⁺) cells in GCs. In addition, we used other immunological markers to compare the distribution of centrocytes within GCs of human tonsil and appendix.

2. Materials and methods

2.1. Tissue samples

Frozen normal human appendix specimens were obtained from Children's National Medical Center (CNMC) in Washington, DC. Fresh tissues were embedded in OCT compound at the hospital and placed immediately in a –70°C freezer. Because the availability of frozen specimens was very limited, we also obtained paraffin-embedded archived appendix tissues from CNMC and from Suburban Hospital in Bethesda, MD. An exemption for the collection of these specimens was obtained from the Office of Human Subjects Research at the National Institutes of Health. Fifty-five donor specimens ranging in age from 2 days to 85 years were obtained. Only tissues that were classified as normal on the pathology report were examined. All frozen sections (human and rabbit) were cut at 5 µm whereas paraffin-embedded sections were cut at 6 µm.

Table 1
Primary antibodies used in study

Target molecule	Host	Clone no. or PC Catalog no.	Cells marked/function	Concentration (µg/ml)	Incubation time
A. Anti-human					
CD4	Mouse	RPA-T4 ^a	T-helper/inducer cells	20	30 min
CD4	Mouse	IF6 ^b	CD4 ⁺ cells on paraffin sections	2.5–5	Overnight
CD8	Mouse	HIT8a ^a	A subset of T-cytotoxic/suppressors cells	20	30 min
CD8	Mouse	C8/144B ^c	CD8 ⁺ cells on paraffin sections	7.5	Overnight
CD38	Mouse	HIT2 ^d	B/T-cells subsets, macrophages, dendritic cells	10	30 min
CD77	Mouse	38-13 ^c	Activated B-cells	3	30 min
Ki-67	Mouse	Ki-67&Ki-S5 ^d	Nuclei of proliferating cells	4	Overnight
IgA	Rabbit	PC no. A0262 ^e	α heavy chain	7.3	Overnight
IgD	Rabbit	PC no. A0093 ^e	δ heavy chain	?	Overnight
IgG	Rabbit	PC no. A0423 ^e	γ heavy chain	5	Overnight
IgM	Rabbit	PC no. A0425 ^e	μ heavy chain	35.3	Overnight
B. Anti-rabbit					
CD4	Mouse	KEN-4 ^f	T-helper/inducer cells	10	30 min
CD8	Mouse	12.C7 ^f	T-cytotoxic/suppressor cells	10	30 min
Ki-67	Mouse	Ki-67 & Ki-S5 ^d	Nuclei of proliferating cells	4	30 min
IgA	Mouse	NRBA ^f	α heavy chain	5	60 min
IgG	Mouse	SVB2 ^f	γ heavy chain	20	60 min
IgM	Goat	PC no. 55627 ^g	μ heavy chain	?	60 min

^a PharMingen International (San Diego, CA).

^b Novocastra/Vector Lab. (Burlingame, CA).

^c Coulter/Immunotech (Miami, FL).

^d Boehringer Mannheim (Indianapolis, IN).

^e Dako Corporation (Carpinteria, CA), PC = polyclonal antibody.

^f Spring Valley Lab. (Woodbine, MD).

^g Cappel/ICN Pharm. (Costa Mesa, CA).

Rabbit appendix tissue was obtained from allo-type-defined pedigreed animals maintained by Spring Valley Laboratories (Woodbine, MD) for the National Institute of Allergy and Infectious Diseases. Animals were fed ad libitum. The mid-section of the appendix was sampled from three different animals for each of the following ages: 1 day, 1, 2, 3, 6, 9, and 12 weeks, 6 and 9 months, 1, 2 and 6 or 7 years. Because fewer immunological reagents suitable for paraffin-embedded tissues are available for the rabbit, only frozen tissues were used for immunohistological staining. Tissues were embedded in OCT compound and frozen in either liquid nitrogen or partially submerged in a slurry of dry ice and isopentane.

2.2. Immunohistochemical staining

Primary antibodies used for staining are listed in Table 1. Frozen sections were fixed in acetone for 7 min. Frozen and paraffin-embedded sections were blocked with 5% goat serum for 30 min. Secondary antibodies included either biotinylated goat F(ab')₂ anti-mouse IgG or goat anti-rabbit IgG (Southern Biotechnology) incubated for 40 min. This was followed by streptavidin alkaline phosphatase (AP) conjugate incubated for 30 min (Vector). The substrate was Vector Blue and counterstain was nuclear fast red. For staining CD4, CD8 and Ki-67 on paraffin-embedded sections, a streptavidin peroxidase

conjugate (Vector) was used in lieu of streptavidin AP. Diaminobenzidine (DAB) (Vector) was the substrate and hematoxylin the counterstain. The use of peroxidase or AP was based on which staining method gave the best results for a given fixation method and antigen of interest. Negative controls were immunoglobulins (Southern Biotechnology and Pharmingen) matched according to isotype and concentration. Double staining for CD4/CD8⁺ cells on frozen sections involved the following sequence: anti-CD8, anti-mouse IgG biotinylated, streptavidin AP, Vector Blue, avidin and biotin blocking solutions (Vector), anti-CD4, anti-mouse IgG biotinylated, streptavidin AP, and Vector Red substrate.

Antigenic sites on formalin-fixed paraffin sections were unmasked using one of the following pre-treatments. Tissues stained for IgM and CD antigens were pre-treated by heating for 40 min in 10 mM citrate buffer (pH 6.0) using a microwave pressure cooker (Nordic Ware, Minneapolis, MN) in a microwave oven operated at 900 W. Tissues stained for IgA and IgD were incubated for 5–10 min with 0.05% Pronase (Dako, Carpinteria, CA) following the supplier's recommendations. Ki-67⁺ antigenic sites were unmasked by boiling tissues 10 min in 10 mM citrate buffer (pH 6.0).

2.3. Area measurements

The areas of the FR and GC relative to the total cross-sectional area of paraffin-embedded human appendix sections were determined. The analyses were performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and is available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Thirty human specimens were analyzed; ages ranged from 2 days to 19 years. Specimens from older individuals were not analyzed since this has been reported previously [29]. Specimens were arbitrarily classified into five age groups consisting of five to six specimens in each group. Only transverse rather than oblique sections were analyzed as judged by symmetry.

For comparison, we also calculated the percent-

age of the FR in the rabbit at various ages throughout the life span. Rabbit specimens were arbitrarily divided into eight age groups containing three animals per group. In lieu of GC area, we calculated the area of proliferating cells (proliferation zone) as shown by staining for Ki-67. This was done because the GC is not as apparent in the rabbit as in the human appendix, and because intense cell proliferation is known to occur in the GC although less so in the light zone (LZ) than dark zone (DZ).

2.4. T-cell enumeration

Digitized images were made of appendix follicles viewed at 100 \times . CD4⁺ cells were counted using a grid containing 12 mm² areas to sample several sites in the B-cell FR. Because the B-cell FR is sparsely populated with CD8⁺ cells, all CD8⁺ cells were counted within the FR and the area was determined by NIH Image analysis software. In the rabbit appendix, the GC is largely confined to the base of the follicle as evident by Ki-67 staining (Fig. 4(C)). The demarcation between the dome/corona and base of the follicle was defined as the center of the region where adjacent T cells areas flanked the follicle. In the human, the border between the GC and mantle zone of the follicle is clearly evident based on cell density and size (Fig. 1(B)). Three rabbit appendices and four to five different human appendices were analyzed for each age or age group. From each tissue, three follicles were randomly selected for counting. The follicles were sectioned through the center so that each included the base and tip of the dome.

3. Results

3.1. Morphology

We first compared the lymphoid development of the rabbit and human appendix using hematoxylin and eosin-stained cross sections. Fig. 1 shows representative ages of human and rabbit appendices across the life span. In both human and rabbit appendices, lymphoid follicles were

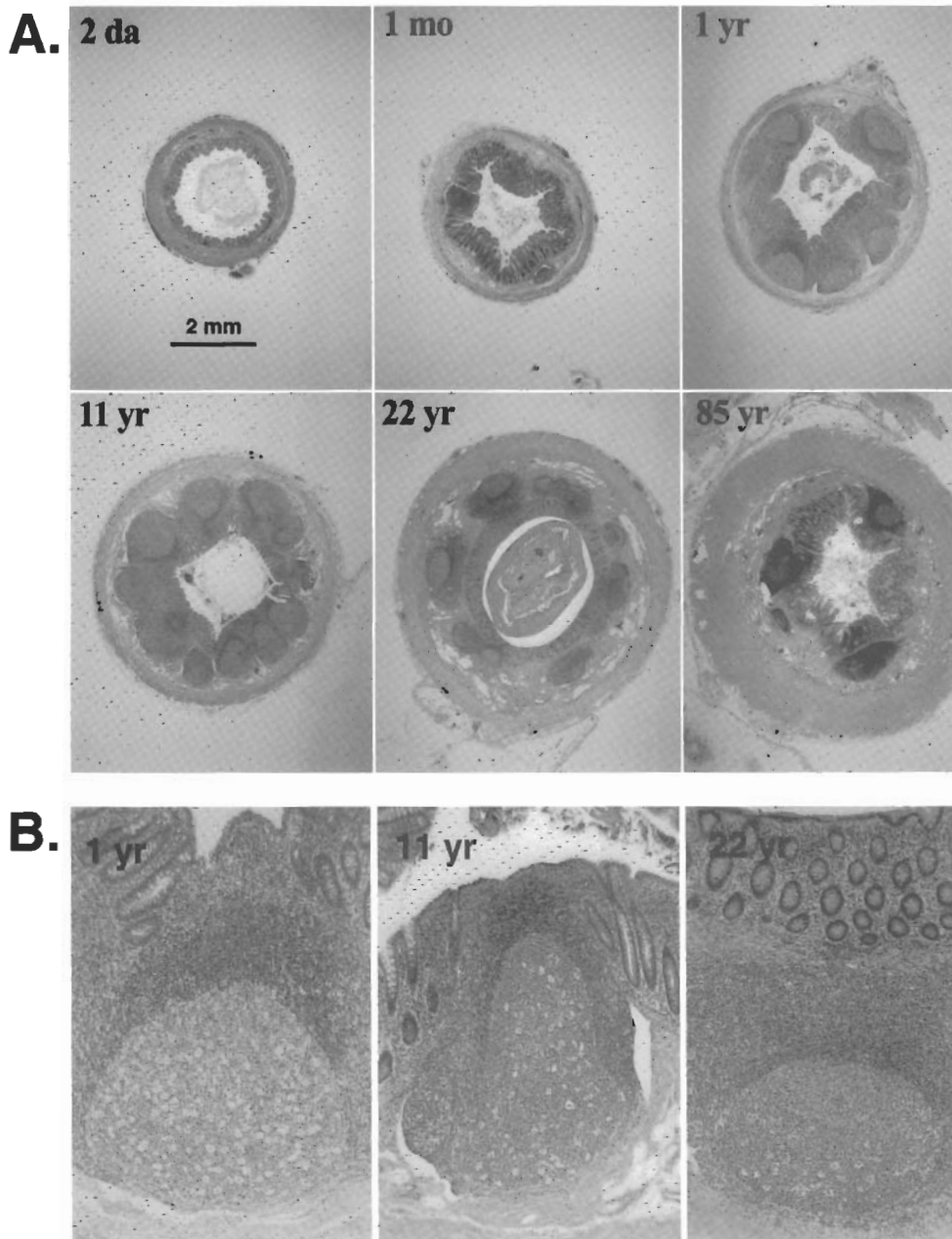
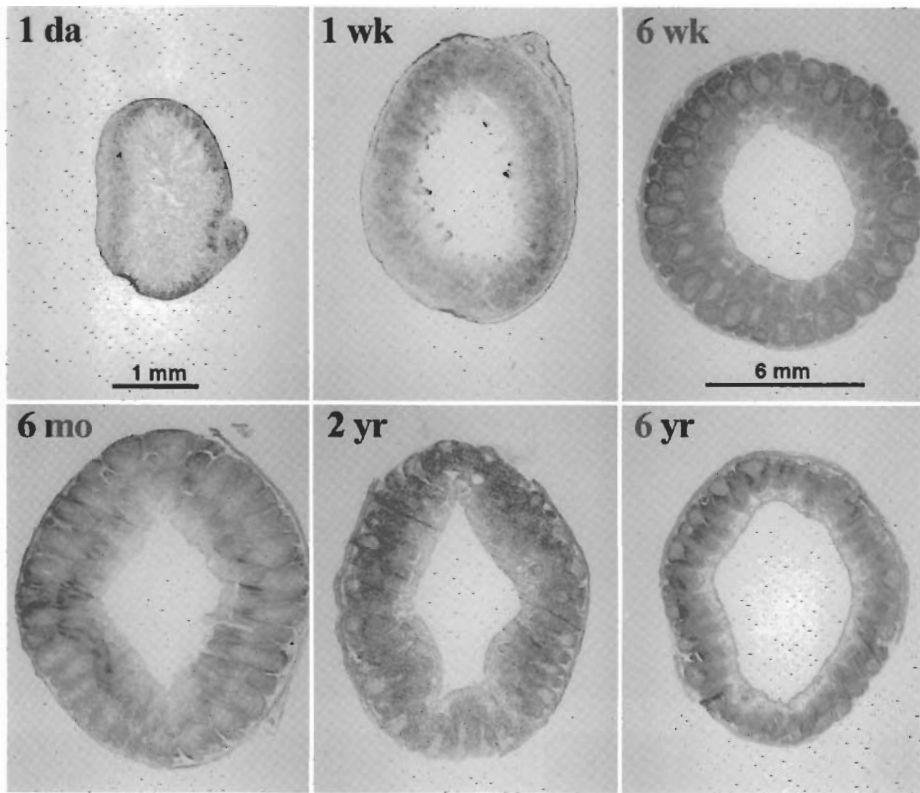


Fig. 1. Cross sections of normal human (A) and rabbit (C) appendices at various representative ages. Bars indicate scale (2 mm scale for all human ages, 1 mm for rabbit aged between 1 day and 1 week, 6 mm for rabbit aged between 6 weeks and 6 years). Paraffin-embedded (A, B, D) and frozen (C) sections were stained with hematoxylin and eosin. Note the near lack of lymphoid follicles in the neonates followed by rapid lymphoid development during youth and gradual decline in adulthood. The follicles of the human appendix become more elongated in youth and more spherical with age (B, original mag. 100 \times). The rabbit appendix follicles also become less elongated, more rounded with advance age and resemble that of the young jejunal Peyer's patch (D, original mag. 50 \times).

C.



D.

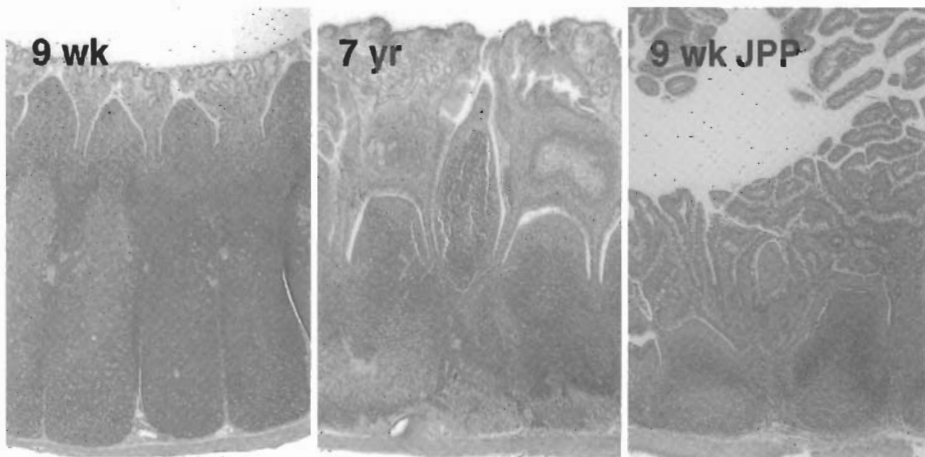


Fig. 1 (continued)

just beginning to form at birth. GCs became evident at about 1 month in human appendix and at 1 week in rabbit appendix. Most lymphoid developments occurred within the first year in the human appendix and within the first 12 weeks in the rabbit appendix. The mean outer and inner diameters of the adult rabbit appendix are much greater than those of the human (10.5 and 6.5 mm, respectively, for outer diameter). Lymphoid follicles began to atrophy in the teen years in human appendix and by young adulthood in rabbit appendix. The follicles became smaller and fewer in number whereas the connective tissue and smooth muscle increased. The appendix did not entirely involute. GCs were still present in advanced age as evident in the 85-year-old human and 6-year-old rabbit specimens (Fig. 1(A) and (C)). The shape of the follicles changed with age. In the human appendix, the developing follicles gradually became more elongated late in childhood and more spherical in adulthood (Fig. 1(B)). In the rabbit appendix, the follicles also gradually lengthened up to 9 months of age and then regressed in length (Fig. 1(D)). The follicles in the rabbit appendix, however, are much more elongated than in the human; the former resemble the shape of an Erlenmeyer flask with a dome top. In advanced age, the follicles lose their flask shape (Fig. 1(D), 7 years) and resemble the more spherical shape of jejunal Peyer's patches (JPP) of young animals (Fig. 1(D), 9 weeks).

To determine at what age the lymphoid tissue is most developed in human and rabbit appendices, we calculated the percentages of the FR and GC relative to the cross-sectional area for various age groups (Fig. 2). In the human appendix, cross-sectional area increased gradually from birth through the teen years whereas the percentage of the FR increased rapidly within the first year of life and changed little up through the teen years. GC area was absent or scant during the first 12 weeks of life, peaked later in childhood, and declined during the teen years. It had been reported previously that in the human appendix, aggregate lymphoid tissue is scarce before birth [30] and steadily declines after the first decade of life [29].

Compared to the human, the rabbit appendix contained much more lymphoid tissue (Fig. 2). The peak percentage of the FR was 81% in rabbits at ages 6–9 months. In children, the highest percentage of the FR was 29% which occurred at ages 1–11 years. In humans the GC was highly Ki-67⁺ (Figs. 3(A) and 4(A)) and resembled the proliferation zone in the rabbit appendix (Fig. 4(C)). However, the percentage of the proliferation zone peaked at over 50% in the rabbit at the age of 6 weeks whereas the highest percentage in the human appendix was 10% during the period from 1 to 11 years of age (Fig. 2(A)).

3.2. Immunohistochemistry — human

To further compare human and rabbit appendix development, we stained for Ig classes, GC regions and T cells subsets. When antibodies of interest would not stain paraffin-embedded sections, frozen sections of human appendix were stained. The GC was clearly identified by antibody to CD38, a GC marker, and by anti-Ki-67; CD77 was used to identify centroblasts within the GC [31] (Fig. 3(A)). Note that the pattern of Ki-67 staining closely matches with that of CD38. CD77 also stained throughout the GC although less intensely than CD38 and Ki-67. Similar results were found using human tonsil (Fig. 3(B)).

Frozen human appendix sections from a 4-year-old donor were also stained for Ig classes to compare with staining of paraffin-embedded tissues (Figs. 3(C) and 4(A)). Anti-IgM and anti-IgD staining patterns were similar whereas staining with both anti-IgA and anti-IgG was more intense on frozen sections (the anti-IgG reagent did not stain paraffin-embedded tissues). Staining was most intense with anti-IgA and least with anti-IgG. Anti-IgA and anti-IgG stained the GC only, anti-IgD stained the mantle zone exclusively, and anti-IgM stained both the mantle zone and GC.

Staining of paraffin-embedded sections of human appendix from representative ages ranging from 4 days to 85 years is shown in Fig. 4(A) Anti-IgA staining was negative until after the first week of life. Anti-IgA stained the

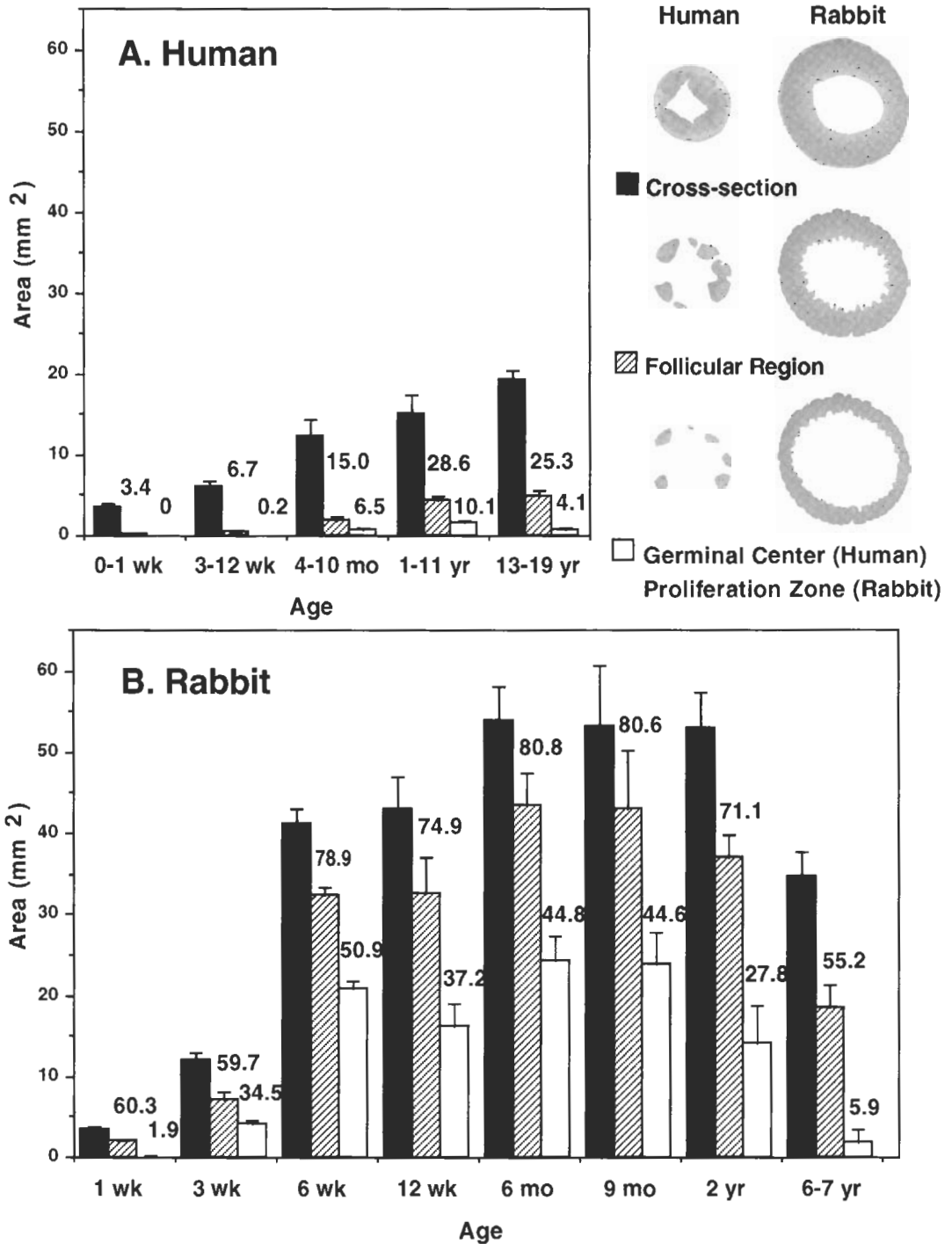


Fig. 2. Lymphoid tissue area in developing human (A) and rabbit (B) appendices. The mean percentage of the FR and GC or proliferation zone area relative to the cross-sectional area is shown for various ages. Sample tissue areas are shown in the legend. Bars represent mean \pm SE. The area of proliferation as determined by Ki-67 staining is shown in lieu of GC area for the rabbit appendix.

GC faintly across donor ages except in the 80-plus-year-old samples. A few IgA⁺ pre-plasma cells were found in the GC and numerous plasma cells were found in the lamina propria including that of the oldest specimens. Anti-IgM and anti-IgD stained follicles from the youngest to the oldest specimens examined, but was less intense in the latter. By 4 months, IgD⁺ mantle zones were evident. Ki-67⁺ cells were found throughout the GC but were most numerous at the base.

Prior to GC development, Ki-67⁺ cells were scattered throughout the follicle. The entire GC was highly positive for Ki-67 even in advanced age.

3.3. Immunohistochemistry — rabbit

In the rabbit appendix, staining of IgM⁺, IgA⁺ and IgG⁺ lymphocytes was negative at day 1 although high background staining of the lamina propria occurred with anti-IgG (Fig. 4(C)). By 1 week, follicles stained lightly for IgA, IgM and IgG in the dome and base. Staining for the three Ig classes became more intense by 6 weeks. By 2 and 7 years, Ig staining was less intense, less prevalent in the dome, and more restricted to the center of the LZ. At all ages save for 1 week, IgA staining was darkest.

Ki-67⁺ cells were scattered in the 1 day rabbit appendix and became concentrated in GCs by 1 week (Fig. 4(C)). Ki-67⁺ cells densely populated the base of the follicle and were less numerous in the central LZ as shown in the 6-week- to 7-year-old appendix (Fig. 4(C)). In the oldest rabbits, Ki-67⁺ cells occupied a much smaller proportion of the follicle (Fig. 4(C)).

3.4. Numbers of T cells in B-cell follicular regions

Human and rabbit appendices from six age groups were examined to determine the cell density of CD4⁺ and CD8⁺ cells in the B-cell rich FRs (Fig. 5(A) and (B)). Cells were counted in the mantle zone and GC in the human, and in the dome/corona and base of the rabbit appendix follicles. The base of the rabbit B-cell FR approximates the human GC (as suggested by staining with Ki-67). A few T cells were found in the B-cell rich FR of the 1-week-old rabbit appendix but were not included in Fig. 5 because of difficulty in distinguishing the dome/corona and base of the follicle at that age.

In the human appendix, T-cell areas became evident by 1 month of age. CD4⁺ and CD8⁺ cells were present in the GC at the earliest time point examined. CD4⁺ cells were far more numerous in the GC than in the mantle whereas the converse was true for CD8⁺ cells (Fig. 5(A)). In the GC, CD4⁺ cell density was low during the

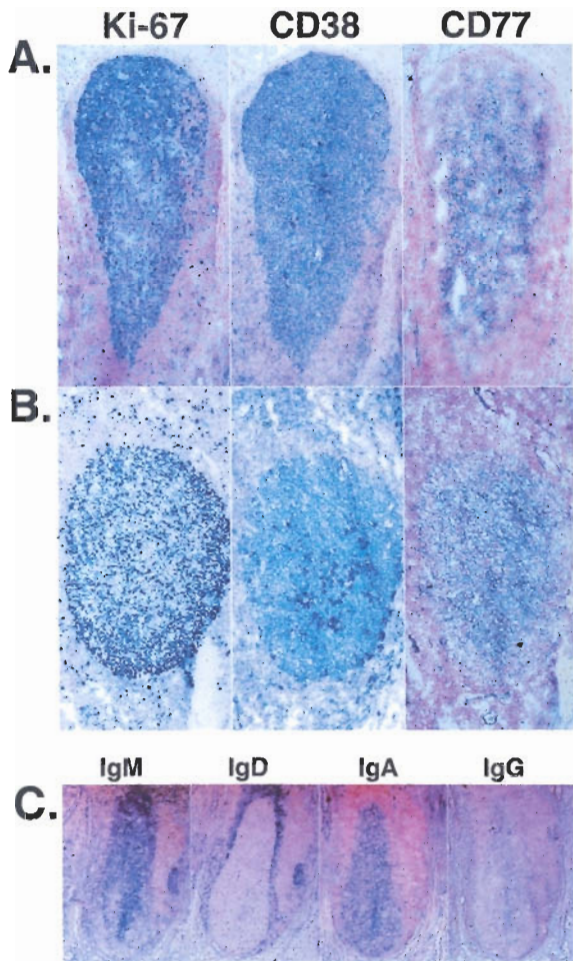


Fig. 3. Immunohistochemical staining in frozen sections of human 4-year-old appendix (A, C) and 8-year-old tonsil (B). GCs were stained with antibodies to Ki-67, CD38 and CD77 (A, B). Appendix germinal centers were stained for the four Ig classes (C). Positive staining is shown in blue (original mag. 100 \times).

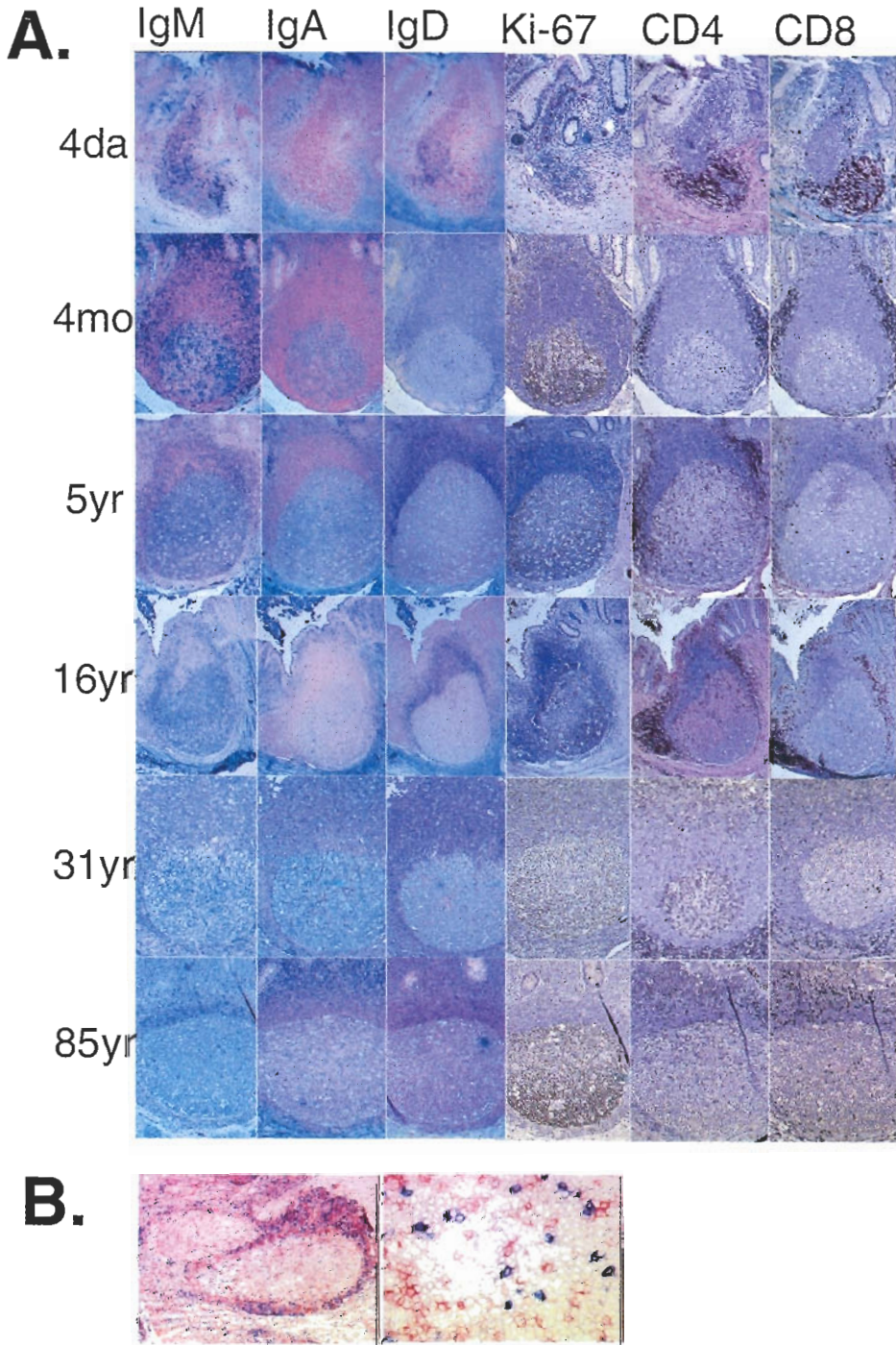


Fig. 4. Immunohistochemical staining of paraffin-embedded sections of human (A) and frozen sections of rabbit (C) appendices at various representative ages. Positive staining is shown in blue for all rabbit tissues and for human Ig classes. Human CD4⁺, CD8⁺ and Ki-67⁺ cells are stained dark brown. Double staining for CD4⁺ (red) CD8⁺ (blue) cells in 4-year-old frozen human appendix (B) indicated few if any cells were double positive.

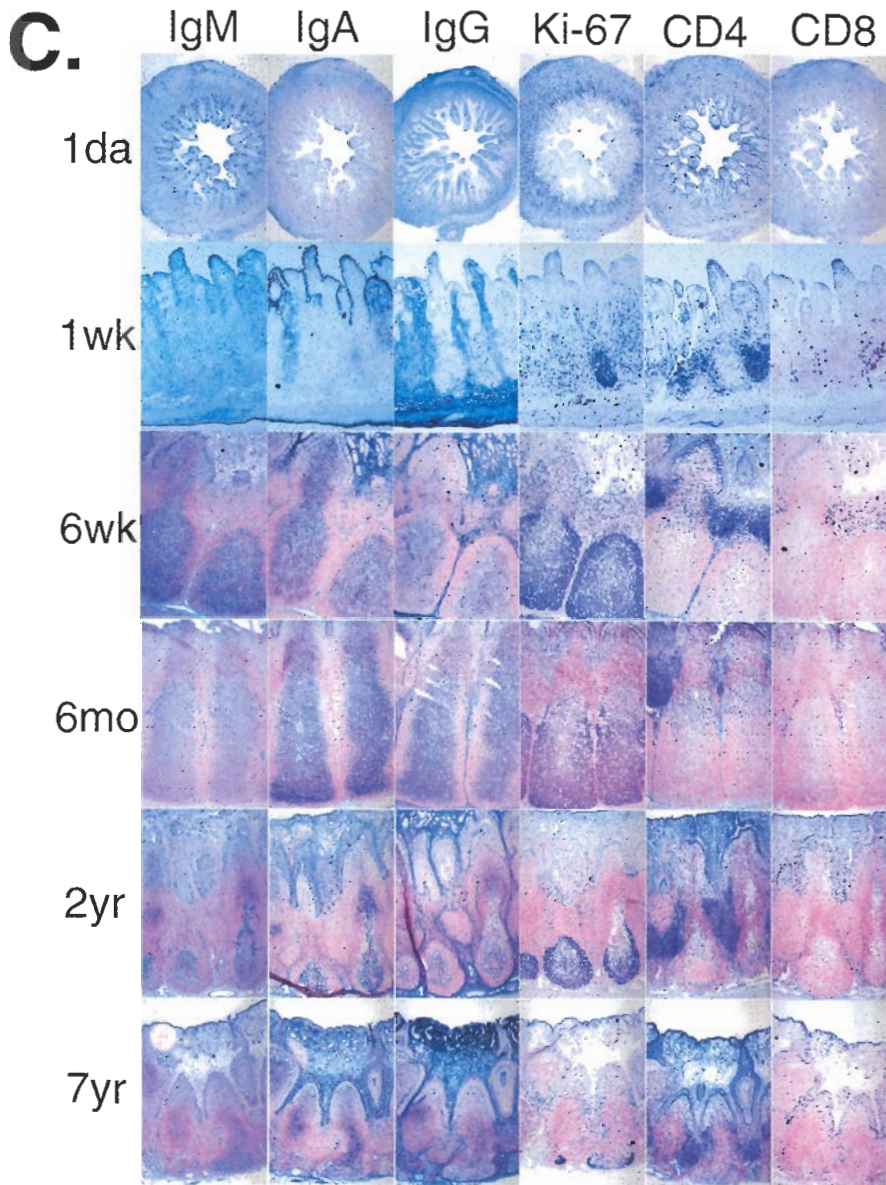


Fig. 4 (continued)

first 3 months of life, rose sharply between 4 and 10 months of age, and remained fairly stable through the rest of life. In the mantle, CD4⁺ cells increased gradually until adulthood. CD8⁺ cell density in the B-cell FR was more variable than CD4⁺ cell density. Even within the same cross section, CD8⁺ cell counts were highly vari-

able between follicles. Mantle CD8⁺ cells increased greatly in the oldest relative to youngest age group whereas an increase in GC CD8⁺ cells was less pronounced. Double staining indicated few, if any, cells were CD4/CD8 positive (Fig. 4(B)).

In the rabbit, T cells were scattered in the 1-

day-old appendix but by 1 week, T-cell areas were visible (Fig. 4(C)). As in the human appendix, T cells were found in the GC as soon as GCs developed. At 1 week, CD4⁺ and CD8⁺ cell densities in the dome/corona and base collectively were 809 ± 52 cells/mm² and 94 ± 15 cells/mm², respectively. CD4⁺ cells were more numerous in the dome/corona than in the base which is opposite the pattern observed in human appendix follicles (Fig. 5(A) and (B)). In the rabbit, CD4⁺ cells in the base of the follicles increased greatly and consistently throughout life in contrast to human CD4⁺ cell density in the GC which reached a high plateau early in life. In the dome/corona of the rabbit follicles, the increase of CD4⁺ cells throughout life was less dramatic and less uniform than in the base of the follicle. Numbers of CD8⁺ cells were low early in life and increased greatly late in life. Although observed in both human and rabbit, this increase

was more consistent across age groups in the rabbit. In rabbit, CD8⁺ cell counts were greater in the dome/corona than in the base just as they were greater in the mantle zone than in the GC of the human appendix. CD8⁺ cells were most prevalent in the dome perimeter and in the dark zone at the base of the follicle (Fig. 4(C)).

4. Discussion

The rabbit appendix is an important site for early development and diversification of the B-cell repertoire. Because of this functional similarity to the avian bursa of Fabricius, this study examined to what extent the developing human and rabbit appendix share bursa-like morphological and immunohistological features. We compared the lymphoid morphology and staining pattern for Ig classes, proliferating cells, the GC

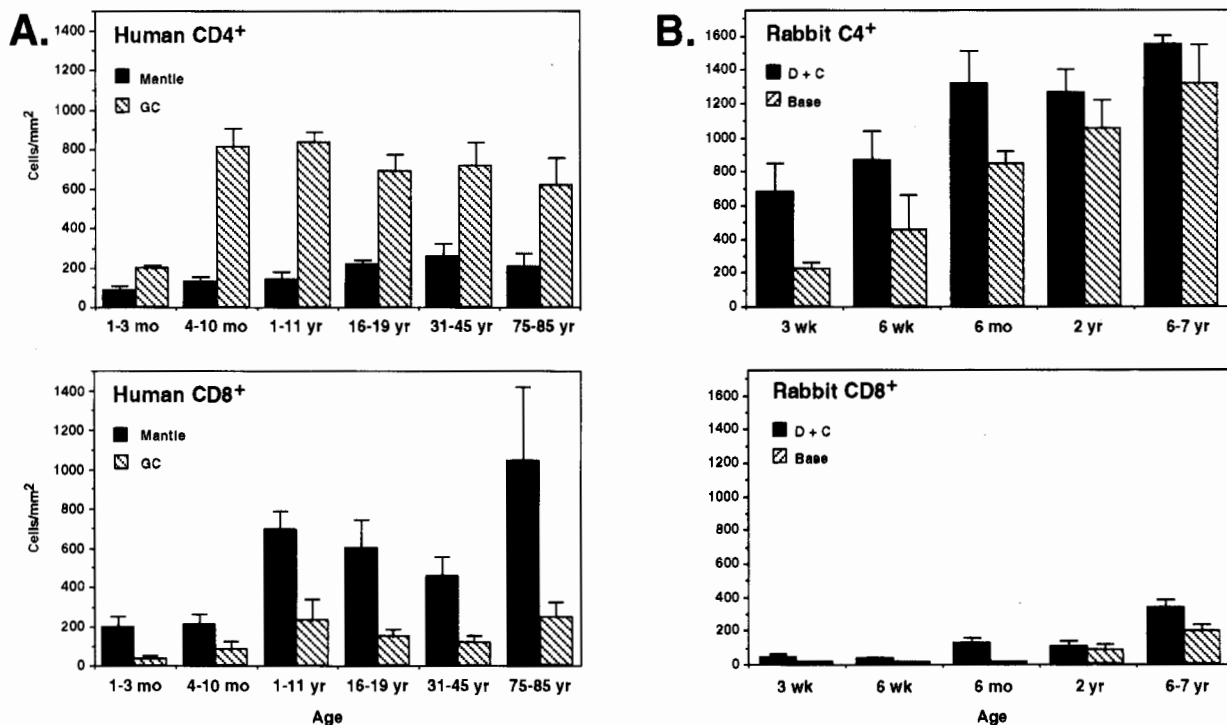


Fig. 5. Density of CD4⁺ and CD8⁺ cells in the B-cell FRs of human (A) and rabbit (B) appendices at various ages. Bars represent mean \pm SE. Cells were counted separately in the dome/corona (D + C) and base of the B-cell FR in the rabbit, and in the mantle zone and GC of the human appendix.

Table 2
Summary comparison of human and rabbit appendices and chicken bursa

Trait	Chicken bursa	Rabbit appendix	Human appendix
Anatomic location/ontogeny	Dorsal wall of cloaca/cloacal epithelium invaginates [38]	End of cecum/intestinal epithelium invaginates [38]	End of cecum/intestinal epithelium invaginates [39]
Effect of neonatal bursectomy/appendectomy or GALT removal	Severe impairment of antibody responses and reduced plasma cell numbers.	Reduced antibody responses, repertoire diversity, and plasma cell numbers	Unknown
Mechanism of primary diversification	Gene conversion/somatic hypermutation	Gene conversion/somatic hypermutation	Unknown whether it occurs
Basic morphology	Has numerous elongated follicles each with a follicle-associated epithelium and a B-cell rich region.	Similar to bursa except young appendix has Erlenmeyer flask shape	Similar to bursa and rabbit appendix except has fewer and less elongated follicles
Time of lymphoid development	Begins in ovo [40]. Driven by microbial flora after hatching [37]	Begins largely after birth [41] Driven by microbial flora [42]	Begins largely after birth [41] Driven by microbial flora [43]
Age-related changes in morphology	Follicles gradually increase in size, regress at sexual maturity and nearly involute completely 2 months later [12,44,45]	Follicles gradually increase in size until young adulthood and then regress but do not involute completely	Same as in rabbit except peak follicular development occurs in late childhood
Structure of follicle	DZ ^a in perimeter, LZ ^b in center. Connective tissue layer between DZ and LZ	Same as in bursa except no connective tissue layer between DZ and LZ	DZ in base, LZ in apical region
Ig staining	IgM dominant (0–11 weeks)	IgA dominant (1 weeks and older)	IgA dominant (4 years).
T cells	No T-cells areas except in diffusely infiltrated area. Very few T-cells in follicles (all ages)	T cells areas. T cells also in GCs, proportions increase with age	T cells areas. T cells also in GCs, proportions do not increase with age except during first few months of life

^a DZ = dark zone.

^b LZ = light zone.

structure, and T-cell numbers in appendix samples at various ages across the life span. In the young rabbit, the primary pre-immune repertoire depends upon intact functional appendix and other GALT. Removal of appendix and other GALT in young rabbits has been shown to reduce the diversity of the antibody repertoire, levels of serum and mucosal antibodies, numbers of plasma cells and circulating lymphocytes [7,14–16]. Similarly, bursectomy results in a marked reduction in plasma cell numbers and antibody responses [32–34]. It is currently unknown whether primary diversification occurs in the human appendix or whether appendectomy affects mucosal antibody levels. Studies in our laboratory are addressing these questions. We conducted the comparisons summarized in Table 2 as a prelude to molecular analyses of whether the human appendix functions in developing the primary immune repertoire.

In mammals and birds, B-cell differentiation begins with VDJ recombination in central lymphoid organs [35,36]. In some species, however, primary diversification of the B-cell antibody repertoire continues in the periphery via gene conversion and/or somatic hypermutation. These processes occur in the rabbit appendix, chicken bursa, and SIPP and are affected by foreign antigens [1–7,37]. Thus primary diversification is considered 'pre-immune' whereas secondary is 'immune'.

4.1. Morphology

Table 2 summarizes and compares various traits of human and rabbit appendices with those of the avian bursa. Historically, the appendix was considered a candidate for a mammalian bursal equivalent partly because the histology and elongated shape of the appendix follicles resemble to that of the bursal follicles [38,46]. The appendix follicles also resemble those in the SIPP [47]. In old age, the morphology of the rabbit appendix follicles resembles the JPP (Fig. 1(D)). Although the change in morphology could reflect a change in function from a primary to secondary lymphoid organ, it may merely reflect the degree of lymphoid development or atrophy.

Neither the human nor the rabbit appendix atrophied completely in adulthood although the GC area was sharply reduced in advanced age (Fig. 1). This implies that the appendix functions immunologically throughout life although this activity gradually wanes beginning in the teen years in man and young adulthood in rabbits. In contrast, involution of the bursa begins at sexual maturity (four to four-and-a-half months of age) and is nearly complete by 6 months of age [12,45]. The SIPP begin to regress at 3–4 months of age and nearly disappear by adulthood [11].

The adult human and rabbit appendices are approximately the same length (about 9 cm [39,41]) but the width and relative amount of lymphoid tissue is considerably greater in the rabbit (Fig. 2). In the rabbit, the appendix has been estimated to comprise half of GALT [48]. It is unknown what percentage of human GALT is in the appendix. Because of its large appendix, Peyer's patches and sacculus rotundus, it is likely that the rabbit has proportionately more GALT than man. Thus comparison of the relative immune importance of the rabbit and human appendices based on size may not be appropriate. Although the human appendix contains considerably less lymphoid tissue than that of the rabbit, it does contain numerous lymphoid follicles. As in the rabbit, the human appendix is also strategically located near the junction of the small and large intestines where it can encounter the first contents of the large bowel, which is rich in microbial antigens. It is possible that the appendix may significantly affect antibody production in the gut just as tonsils do in the upper respiratory tract; tonsillectomy has been shown to reduce antibody levels in serum, saliva and nasopharyngeal washes [49–53].

4.2. Follicular structure

The follicles of human and rabbit appendices have some histological differences which may reflect a difference in function. In the human, the GC can be easily identified because of its lesser cell density relative to the rest of the follicle whereas in the rabbit, the GC is less obvious (Fig. 1(B) and (D)). The DZ of the human GC is

composed primarily of rapidly dividing centroblasts on the basal side whereas the LZ contains mostly resting centrocytes on the apical side (Fig. 6(A)). In the rabbit GC, the DZ lies in the perimeter whereas the LZ is in the center (Fig. 6(B)). In the human appendix we could not distinguish these regions using anti-CD77, which preferentially stains centroblasts. Perhaps this separation of centrocytes and centroblasts may be more temporal than spatial and thus depend on the reaction cycle of each GC [54]. The structure of the human appendix GC is similar to the mouse Peyer's patch except for the fact that the former is more elongated [55]. In contrast, the GC structure of the rabbit appendix resembles the follicular structure of the bursa and SIPP [46,47]. The latter organs have a medulla where cell density is less than that of the surrounding cortex. The bursa, however, is unique in that a capillary network and basement membrane separates the medulla from the cortex (Fig. 6(C)) [12,46].

4.3. Immunoglobulin staining

Human and rabbit appendices are similar in that IgA staining intensity was greatest among Ig classes on frozen sections (Figs. 3(C) and 4(C)) although not on 1-week-old rabbit and paraffin-embedded human sections. This observation agrees with previous findings that both rabbit and human appendices are enriched sources of IgA B-cell precursors [25,26]. In contrast, IgM is

the dominant Ig class expressed by bursal B cells throughout the functional life of the bursa [37]. Human and rabbit appendices differ, however, in that IgA and IgG stain merely the GC in man (Fig. 3(C)), and both the dome/corona and GC in rabbit (Fig. 4(C)). This suggests that the dome/corona region may play a different role in rabbit than in man. Although Ig staining intensity appeared to wane with age, Ki-67 staining did not; however, the total GC area and proliferation zone were smaller (Fig. 4(A) and (C)). These results suggest that the GC and proliferation zone, which is composed of mostly B cells, is proliferating as much in the old and as in the young appendix.

4.4. T-cells in germinal center

Both the human and rabbit appendix contained significant numbers of T cells in the B cell FRs but the developmental distribution of T-cells in the human and rabbit appendix differed. Except for the first three months of life when the GC is in an incipient stage of development, the young human appendix contained relatively more T cells than that of the rabbit (Fig. 5). Contrary to the report of Weinstein et al. [6], we did find significant numbers of T cells in the B-cell FRs in the rabbit at an early age of 1 week. We attribute this discrepancy to greater sensitivity of the staining methods we used. The numbers of T cells, however, increased with age, particularly CD4⁺ T cells in the GC; this increase coincided

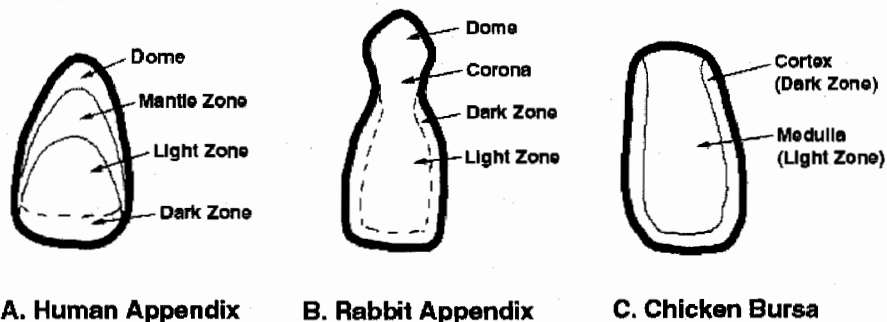


Fig. 6. Follicular structure of human and rabbit appendices, and chicken bursa. The light and dark zones in human and rabbit appendices comprise the GC.

with a morphological change in which the appendix follicles in an old rabbit resembled that of the JPP of a young rabbit as observed by Weinstein et al. [6]. These findings suggest that the young rabbit appendix also functions in secondary diversification of the immune repertoire and that this function increases with age. Thus the transformation of the rabbit appendix from a primary to secondary lymphoid organ as proposed by Weinstein et al. [6] may actually be a matter of degree rather than a complete change in function. The observation that the number of T cells was relatively higher and more stable during development in the human than rabbit appendix suggests that the former is less likely to undergo a transformation in function and more likely to function in secondary diversification throughout life. In contrast to the rabbit and human appendix, the bursa and SIPP follicles contain relatively few T cells [9,11]. In a small region of the bursa, there is a diffusely infiltrated area of lymphoid cells which contains many T cells but this area is histologically distinct from the rest of the bursa [9].

It is not known whether primary diversification of the immune repertoire occurs in adult GALT of man or rabbit. However, the finding of B cells with germline and recently diversified VDJ rearrangements in normal adult rabbit splenic GCs, suggest that primary diversification might occur in adult rabbits [56]. Because rabbits use primarily one V_H gene in VDJ rearrangements, the combinatorial diversity is limited [57,58]. These germline rearrangements may be diversified by antigen-driven gene conversion which may lead to new antigen specificities as a by-product. Thus antigen-driven gene conversion may be a means to diversify the primary B-cell repertoire in peripheral lymphoid tissues of the rabbit [59,60].

In conclusion, the human appendix is similar in many respects to the rabbit appendix, avian bursa and SIPP, yet several differences exist. The GC structure and relative GC T-cell density of the human appendix throughout development differs from that of the rabbit appendix and chicken bursa which suggests that the human appendix is more similar to a peripheral than

central lymphoid organ. If the human appendix did play a role in primary diversification of the antibody repertoire, it may occur during the first few months of life when the GC T-cells number is low. However, it is possible that peripheral lymphoid organs share some central lymphoid function since the distinction between a peripheral and central lymphoid organ may exist on a continuum rather than as a dichotomy [36]. Whether this is true for the human appendix and other GALT warrants further investigation. Molecular studies involving the sequencing of genes encoding antibody heavy and light chain are planned to determine whether the appendix of man plays any role as a primary lymphoid organ.

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