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THE APPENDIX FUNCTIONS AS A MAMMALIAN BURSAL EQUIVALENT IN THE DEVELOPING RABBIT

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ABSTRACT

In this paper we present genomic DNA sequence and histological evidence that the appendix is a site of diversification of the rabbit's primary antibody repertoire. By 6 weeks after birth, the B cell follicular regions of the rabbit appendix and the distribution of the resident lymphoid cells bear a strong morphological resemblance to similar regions within two primary lymphoid tissues, the chicken bursa and the sheep ileal Peyer's patch. However, similarities between the rabbit appendix, chicken bursa and sheep ileal Peyer's patch end as these animals reach adulthood. The rabbit appendix undergoes morphological and cellular distribution changes as it matures taking on the appearance of a secondary lymphoid tissue, while the sheep ileal Peyer's patch and the chicken bursa both involute. We determined DNA sequences of PCR amplified rearranged variable region genes from germinal center B cells of 6 week old rabbits isolated from several different appendix dark zones and light zones. There was a trend toward a higher degree of diversification from the germ-line VH gene DNA sequence in dark zones than light zones. It is likely that both gene conversion and somatic hypermutation are responsible for the nucleotide changes we observed. Our findings suggest that the rabbit appendix functions as a mammalian bursal equivalent early in development. As the rabbit matures, the appendix appears to evolve into a secondary lymphoid tissue resembling secondary GALT in appearance and possibly in function.

INTRODUCTION

Productive rearrangement of three distinct genetic elements, VH, DH, and JH, at the genomic DNA level is necessary before a heavy chain variable region can be transcribed and translated. The rabbit contains approximately 100 distinct VH genes, all of which belong to the VHIII gene family. Of these VH genes, the one located at the 3' end of the locus, VH1, is used by rabbit B cells 80-95% of the time (1, 2). This preferential utilization of one VH gene makes it possible to analyze and sequence the rearranged heavy chain variable region DNA in most rabbit B cells irrespective of the cells' antigen specificity. In addition, Becker and Knight have shown that diversification of this rabbit VH sequence can occur through gene conversion (1).

B cells mature over time through several stages of development within various lymphoid tissues. Antigen specific responses take place within secondary lymphoid tissues such as the spleen, lymph nodes, and jejunal Peyer's patch (JPP). The follicular regions in which these reactions occur are known as germinal centers (GC) (3). While in GC, B cells can go through affinity maturation, which at the genomic DNA level leads to changes in V gene nucleotide sequences by a process known as somatic hypermutation. Another kind of GC

which is not necessarily antigen specific can occur in primary lymphoid tissues like the chicken bursa and the sheep ileal PP (SIPP), both of which are gut associated lymphoid tissues (GALT). These GC are concerned with the diversification of the antibody repertoire of B cells which have already gone through DNA rearrangements utilizing a limited number of V genes. The mechanism used to alter the DNA sequence of light chain variable region genes of SIPP B cells is somatic hypermutation (4); the rearranged VH and VL genes in the chicken bursa are diversified by both gene conversion and somatic hypermutation (5). GC of secondary lymphoid tissues contain CD4 T cells, while those of primary lymphoid tissues are not (6).

During the 1960's several groups proposed that the rabbit appendix, a GALT, might be a mammalian bursal equivalent. Contributing to this belief were experiments in which appendectomized rabbits were found to have lower antibody titers in response to antigen challenge, fewer circulating lymphocytes in the peripheral blood and reduced lymphoid development in peripheral lymphoid tissue. The observed decrease in the B cell pool could not be solely explained by the loss of B cells located in the appendix at the time of its removal (7-10). In this study we present new evidence that the rabbit appendix appears to be a primary lymphoid tissue which acts as a site where the initial diversification of the rearranged VH1 gene takes place. We have found that early after birth the rabbit appendix has many morphological and cellular distribution similarities with the chicken bursa and the SIPP. In addition, we have found that rabbit appendix GC B cells are probably changing their genomic DNA variable region sequences by both somatic hypermutation and gene conversion. However, unlike the chicken bursa and SIPP, the rabbit appendix does not involute by 8-14 months. Instead the rabbit appendix undergoes several changes, one of which may be a change in function.

MATERIALS AND METHODS

Animals

Mixed breed rabbits of the VH1a2 (F-I) haplotype were bred and raised in our own allotype derived pedigreed colonies. Rabbits were sacrificed at 1 day; 2, 4, 6, and 9 weeks; 4, 5, and 9 months; and 1, and 4 years and relevant tissues removed.

Immunohistochemistry

Tissues were frozen in OCT compound and 7 µm sections cut on a cryostat. Tissues were allowed to air dry, and stored in a desiccator cabinet until used. Sections were stained using a previously described avidin biotin method (11). When the primary reagents were non-biotinylated mouse anti-rabbit IgM, and mouse anti-rabbit IgA monoclonal antibodies, a second step antibody was used, biotinylated goat anti-mouse IgG (Southern Biotechnologies, Birmingham, AL). In addition we used biotinylated mouse anti-rabbit CD4 and biotinylated mouse anti-rabbit CD8 monoclonal antibodies (Spring Valley Laboratories, Sykesville, MD).

PCR Amplification and Sequencing of Rabbit Appendix B Cell Variable Region Genes

Germinal center cells from semi-thin sections of 6 week old rabbit appendix stained with succinylated wheat germ agglutinin, were isolated using an Eppendorf micromanipulator and DNA extracts were prepared from isolated cells (12). This was followed by two sets of PCR amplifications with Taq DNAP (Perkin Elmer Cetus, Branchberg, NJ) using primers for the leader exon and a J consensus region, followed by hemi-nesting with primers specific for a highly conserved sequence in framework region 2 and the J consensus region. PCR amplified DNA was purified, cloned into pUC 18, and used to transform library efficiency DH5α (BRL, Gaithersburg, MD). Positive bacterial clones were isolated and their DNA sequenced by the dideoxy chain termination method, labeling with ^{35}S-dATP and using the Taq Track (Promega Biotech., Madison, WI) or the Circumvent DNA sequencing kit (NEB, Beverly, MA).

RESULTS

Histological Analysis of Rabbit Appendix Development

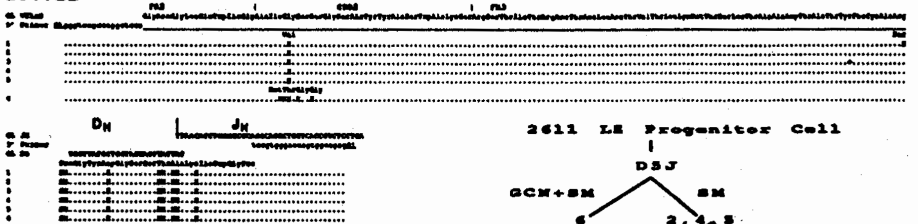
At one day after birth the rabbit appendix is populated by IgM positive B cells, but contains no organized follicular lymphoid structures, in contrast to the chicken bursa and the SIPP. GC can be detected by 2 weeks after birth and these GC contain both IgM and IgA positive B cells. CD4 positive, but not CD8 positive T cells are located in the interfollicular regions of the rabbit appendix at two weeks after birth. However, no CD4 positive T cells were detected in GC or the dome regions of the rabbit appendix, a feature associated with GC of primary but not secondary lymphoid tissues. The rabbit appendix reaches its maximal size by 6 weeks after birth. At this time, CD4 positive T cells are detected in the interfollicular regions of the rabbit appendix, but these cells continue to be absent from GC and the dome regions where many IgM and IgA positive B cells can be found. The GC of the 6 week old rabbit appendix is very similar in morphology to GC found in the chicken bursa and the SIPP. All three lymphoid tissues have GC that are tall and thin, with a dark zone that surrounds the light zone on every side except the luminal one (8, 13). These three lymphoid tissues also have follicle associated epithelium (FAE), the site where antigen is actively transported from the lumen of the gut into the tissue, and pointed dome regions that protrude into the gut lumen (8, 13, 14).

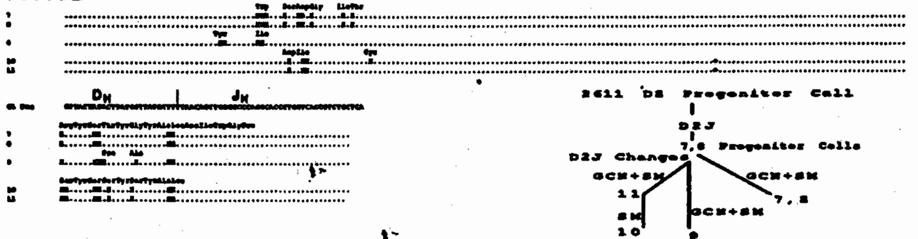
Starting at 9 weeks after birth, and continuing until adulthood the rabbit appendix undergoes several morphological and cellular distribution changes. First, CD4 positive T cells begin to infiltrate the dome region. Then at 4-5 months, CD4 positive T cells are found to occupy the luminal side of GC light zones. Complete population of all regions of GC occurs by 12 months after birth. At the same time that the population of the rabbit appendix B cell follicular regions by CD4 positive T cells commences, morphological changes are initiated. Around 4-5 months after birth, the FAE and dome regions begin to lose their pointed shape and round off with the consequent enlargement of the cellular traffic zones and the FAE in relation to the other regions of the appendix. By adulthood these regions of the rabbit appendix complete their transformations and resemble similar regions of the rabbit and mouse JPP, both of which are secondary lymphoid tissues. The GC also undergo morphological changes during this time as they shrink by 50-60% and broaden out. The net result of these cellular distribution and morphological changes is a lymphoid tissue in the adult that does not resemble the same organ of the young rabbit.

Variable Region DNA Sequence Analysis of Rabbit Appendix GC B Cells

Appendix B cells were isolated from 6 week old rabbits from the two primary compartments of the GC, the dark zone and the light zone, to examine whether DNA sequence diversification of the antibody repertoire was occurring. The rationale for isolating GC B cells at 6 weeks after birth came from our observations that the rabbit appendix GC reached their peak in size, and closely resemble similar sites in both the chicken bursa and the SIPP at this time.

All variable region DNA sequences that we have cloned and sequenced in this study appear to have utilized VH1. Dark zone GC B cells were found to contain rearranged variable regions with a high degree of sequence diversity when compared to the germ-line VH1 DNA sequence (Fig. 1). Most of the base pair changes were found in complementarity determining regions (CDR), but several nucleotide changes were detected in framework regions (FR). In contrast, variable regions from light zone GC B cells were closer to the germ-line VH1a2 DNA sequence. Those light zone VH sequences which contained base pair changes, tended to be less diversified than comparable dark zone B cell sequences (Fig. 1). Both dark zone and light zone GC B cells probably diversify their variable region genomic VH1 DNA sequences through gene conversion and somatic hypermutation. Changes in VH sequences have been found to result in almost exclusively amino acid replacements, with only a few silent substitutions. Finally, within an individual section, dark zones and light zones appear to contain cells which originated from only a few progenitor cells (Fig. 1).





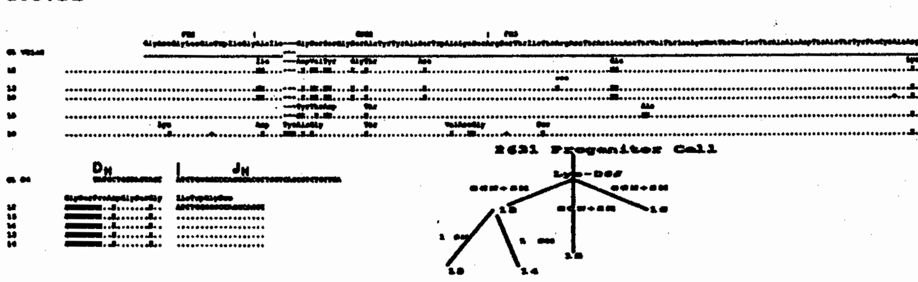


Figure 1. Representative examples of rearranged VH gene sequences of cloned PCR amplified DNA from a light zone (LZ) and dark zones (DZ) of a single GC of a 6 week old rabbit appendix. A full paper with all the sequences from several GC is in preparation. The 5' and 3' primers used in the second hemi-nesting reaction are shown in lower case letters (see Materials and Methods). Evolutionary trees of the clonally related VH gene sequences are shown for each set. Abbreviations are: GCN, gene conversion; SM, somatic mutation. 2631 DZ is from the same GC as 2611 LZ and 2611 DZ, but was taken from a section approximately 7 µm from 2611 DZ. Dots (....) signify identity with germline VH1a2 or known closest DH and JH sequences, dashes (---) signify gaps inserted to align sequences. Almost all DNA base pair changes led to amino acid replacements; Xs represent replacement changes and + silent changes; (***) indicates a change that generated a stop codon. Note the greater diversity of rearranged VH gene sequences from B cells collected from 2611 DZ and 2631 DZ compared to 2611 LZ.

DISCUSSION

Our data suggest that the rabbit appendix is a mammalian bursal equivalent during the early stages of neonatal development. At 6 weeks of age the rabbit appendix has many of the morphological, and cellular distribution characteristics associated with both the chicken bursa and the SIPP. The most important of these is the lack of CD4 T cells in the rabbit appendix GC. The general morphology of the rabbit appendix, with its pointy dome and tall thin GC with dark zones enveloping light zones is very similar to these regions of the chicken bursa and SIPP. In addition, by approximately six weeks of age, all three lymphoid tissues reach their peak in both gross anatomical and GC follicle size, and the rearranged V genes in B cells are undergoing somatic diversification (7, 13).

The rabbit appendix also has several features which appear to make it unique. At birth both the chicken bursa and the SIPP contain GC, while the rabbit appendix has no organized follicular structures. The development of GC in the rabbit appendix appears to be antigen dependent, unlike the chicken bursa and SIPP, which develop GC in the absence of antigen. However, antigen is necessary in order for the GC of the SIPP to reach their maxi-

mal sizes (15). DNA sequences from GC of fetal SIPP have few changes from the germline DNA sequence (4). Therefore, even in sheep the genetic diversification mechanism may be stimulated by exposure of the B cells to environmental antigens. The exact nature of the antigens responsible for driving these GC is unknown, but we have found remnants of bacteria in the rabbit appendix which leads to the suggestion that bacterial antigens, B cell mitogens, or superantigens may play a role. We plan to determine the degree of antigen specificity of B cells undergoing diversification in rabbit appendix GCs in future experiments.

The variable region diversity we find in rabbit appendix GC probably results from both somatic hypermutation and gene conversion with most of the changes localized within the CDR. It is striking that in rabbit appendix GC B cell sequences there is a marked preponderance of amino acid replacement changes. These changes may be indicative of a diversification process by gene conversion, and not affinity maturation by somatic hypermutation because we find very few silent nucleotide changes in the variable region DNA sequences that have been examined. Indeed, antibody diversification and affinity maturation must be different. Affinity maturation is a fine tuning process to yield a better antibody against a specific antigen responsible for initiating an individual GC B cell response. The multiple amino acid changes we see in the rearranged variable region DNA sequences of rabbit appendix GC B cells suggest that the antigen binding specificity of many of these clonally related cells may be changing. Thus, antibody diversification is a more likely explanation of our results. In the future we plan to examine whether a similar diversification process is occurring for light chain genes in the rabbit appendix early in life.

The rabbit appendix goes through a gradual process of change leading to a tissue in the adult animal that has little similarity to the appendix of a neonate. This change is in contrast to the chicken bursa and the SIPP, both of which involute by 8-14 months. The end result of the morphological and cellular distribution changes that the rabbit appendix goes through is the development of a lymphoid tissue that now resembles a JPP, a known secondary lymphoid tissue. The increase in both FAE surface area covering each follicle and the number of cells trafficking through the adult appendix are consonant with the idea that antigen-specific lymphocyte responses are occurring rather than the primary diversification we suggest occurs in the young rabbit appendix. If such functional changes have indeed occurred in the adult rabbit appendix, there may be a stage when primary and secondary lymphoid functions coexist.

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