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# Molecular Characteristics of the Neonatal B-Cell Repertoire

## 1. INTRODUCTION

Development of the neonatal repertoire occurs through a process in which antigen-specific B-cell clones are generated and maintained during adult life or are eliminated early after birth.

Based on the possible random combinations of germ line gene segments (V[D]J and VL-JL) and the random pairing of heavy and light chains that take place in pre-B cells, the potential neonatal B-cell repertoire is greater than 100 million distinct B-cell receptor (BCR) specificities. During the early antigen-independent phase, the repertoire is largely synchronous. However, after birth it quickly achieves an asynchronous pattern because clonal specificity in the neonatal repertoire emerges in a nonrandom fashion. This is based on multiple rearrangements of gene segments encoding the BCR, which will exhibit different specificities.

In addition to genetic elements encoding the BCR, the neonatal repertoire results from the interaction of many other genetic elements, including some that are linked to an Ig allotype and others that are not. This conclusion arises from a study carried out in inbred murine strains *(454–457)*. Studies in F1 mice analyzed antigen-specific clones that induce a polyclonal response and showed that they are codominantly expressed *(454–456)*. Other studies analyzed the oligoclonal responses for certain epitopes and showed that the expression of clonotype is controlled by allotype or other genes. Thus, analysis of influenza hemagglutinin (HA)-clonotype showed that the neonatal repertoire of F1 mice differed from both parents because many parental clonotypes were absent, and novel clonotypes not expressed in the parents had emerged *(457)*. In Balb/c mice, spectrotype analysis of anti-β2-1 fructosan antibodies showed five bands, three of which were predominant. By isoelectrofocusing (IEF) gel analysis, these bands focused in the pH 6.3–6.8 range. In Balb/Cx C57Bl/6 F1 mice and B.C8 mice, the spectrotypes were more heterogeneous than that of either parent, indicating that the expression of *VH* genes encoding β2-1 fructosan-spe-

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cific clonotypes is under the influence of genes encoding allotype-, major histocompatibility (MHC)-, sex-, or color. The gene controlling the diversity of the anti-β2-1 fructosan response was designated *Sr1*. It maps to chromosome 14 in mice *(458)*.

There is some degree of stability and plasticity in the neonatal B-cell repertoire. The stability of the neonatal repertoire can be related to maternal antibodies that clear the antigen to which the newborn is exposed or to intrinsic properties of some neonatal clones to proliferate during postnatal life. This concept is supported by idiotype analysis of B cells producing anti-hen egg lyzozyme (HEL) antibodies after immunization with an lipopolysaccharide (LPS)-HEL conjugate. This experiment showed that the idiotypes of antibodies were similar in newborn mice and adult mice *(459)*, indicating that the development of some neonatal clones is fixed throughout life. In contrast, the analysis of HA-specific clonotypes present after birth showed a lesser degree of diversity at 6 d of age compared to 12–14 d of age. Several clonotypes found in 6-d-old mice were absent in 13-d-old mice, and others were found at high frequency in 13-d-old mice that were not detected in 6-d-old mice *(460)*. These findings could be explained by suppression of some clonotypes soon after birth by maternal antibodies, whereas the occurrence of new clonotypes may be to the result of antigen-dependent expansion of B cells emerging from the bone marrow. Alternatively, the stability or rapid change in the neonatal repertoire may result from genetic programs favoring the extinction or expansion of certain clonotypes during neonatal life.

Antigen exposure of the newborn may result in further development of the neonatal repertoire, leading to diversification of neonatal B cells by other somatic mechanisms such as somatic hypermutation, gene conversion, *N*-region nucleotide deletion, or addition mediated by TdT and receptor editing. The ligand-driven events may have a net effect of preserving and fixing some clones expanded by antigen exposure.

These observations indicate that the neonatal B-cell repertoire should exhibit different molecular characteristics compared to the adult B-cell repertoire. Such differences may consist of alternative utilization of $V_H$ and $V_L$ gene families, biased pairing of $V_H$ and $V_L$ genes, frequency of *N*-addition and/or somatic mutation, and class switching and temporal expression of some *V* genes after birth.

It is noteworthy that these molecular characteristics may be species-specific and may take place in different lymphoid organs in various species. Species-specific differences were identified in comparative immunology studies.

## 2. DIVERSIFICATION OF THE NEONATAL REPERTOIRE IN THE ANURANS

The anurans and mammals diverged at the end of the Devonian period (370 million yr ago). Thus, the study of the diversification of the neonatal repertoire may shed light on what is essential for the immune system of vertebrates in general and what is specific for mammals. For instance, differences in antigen exposure of mammalian embryos, which develop in an antigen-free environment in the uterus, compared to larvae soon after hatching and the transition at the metamorphosis stage when the larval immune system becomes immunocompetent provide precious information for understanding the neonatal B-cell repertoire.

In Xenopus, there are about 100 $V_H$ genes. In larvae, the $V_H$ genes, based on structural homology, were classified into 11 families. The $V_H$ genes recombine with more than 19 $D_H$ and 9 $J_H$ genes. In early stages, the *V[D]*J genes recombine with the *Cμ constant* gene but later can switch to IgY, a homolog of mammalian IgG, and IgX, preferentially produced in the gut. Three genes encode the constant light chain: π, σ, and λ (data reviewed in ref. *254*).

The development of larval B cells is divided into two phases: one starts at d 4 when the $V_H$ genes are rearranged and the first RAG transcripts can be detected. The second phase starts at d 12 and extends to the end of metamorphosis. During this period, the $V_L$ genes are rearranged and the B-cell repertoire begins to diversify. The diversification of the larval B-cell repertoire probably contributes to the ability of the larval immune system to produce antibodies against various antigen. From the time that the spleen becomes visible (d 12), the majority of B cells express sIgM *(461)* and differ from adult B cells in re-expression of the BCR after capping *(462)*.

The diversity of the larval B-cell repertoire through metamorphosis results from recombination of germ line exons encoding the *V* genes rather than via antigen selection. Very few DJ recombinations are abortively rearranged in larvae *(463)*. The diversity of the repertoire results from the utilization of all $V_H$ gene families with the exception of $V_H11$, which is rarely used in adult pre-B cells. A biased rearrangement of $D_H12$ and $D_H16$ with $J_H6$ was observed in pre-B cells, but sequence analysis of the $V_H$ genes of the tadpole at d 30–35 showed that all the rearrangements were different *(464)*. Although no *N*-diversity was observed in the larval repertoire *(464)*, the CDR3 of the developing Xenopus were quite diverse. Most larval and neonatal $V_H$ genes sequenced contain CDR3s encoded by 3–10 codons *(465)*, which is much shorter than in mammals, suggesting that the shorter length may explain the more limited

diversity of the neonatal B-cell repertoire. No receptor editing leading to a second rearrangement of $V_L$ genes has been reported in Xenopus *(466)*.

This information strongly suggests that the diversification of larval and neonatal repertoires in amphibians results mainly from somatic recombination of germ line sequences with little additional somatic diversification by *N*-nucleotide addition or receptor editing.

## 3. DEVELOPMENT OF THE AVIAN B-CELL REPERTOIRE

In birds, repertoire diversification is generated by recombination of a single copy of the $V_H$ ($V_H1$) gene with a D segment that exists as a multigene cluster *(467)*. The $V_H$ gene pairs with a single copy of the $V_L$ ($V\lambda1$) gene. The $V_L$ exhibits rapid diversification by a gene conversion process using a group of pseudogenes as a donor. The first rearrangement occurs during embryogenesis in the pre-B cell. During prebursal rearrangement of D→J and V→DJ, no *N* additions were observed *(467)*. Whereas in mammals there are genetically programmed sequential rearrangements of gene segments (D-J, V-DJ, and $V_L$-$J_L$), in birds the rearrangement of *V* genes is stochastic rather than sequential. Among 100 clones analyzed, several had $V_H$-$V_L$ rearrangements, others only $V_H$, and 5 clones only $V_L$ genes. In these cells, the $V_H$ genes were in the germ line configuration *(468)*. Diversification occurs in the bursal stage after birth, and only the rearranged V genes undergo gene conversion *(469)*. Therefore, although *V[D]*J and $V_LJ_L$ recombination and sIg expression are bursa-independent events, the bursa is required for the diversification of the B-cell repertoire after birth, and at hatching, 90 to 95% of bursal B cells express a BCR. During the bursal stage, after birth, and later in adult life, both $V_H$ and $V_L$ genes undergo gene conversion by transfer of a block of information from pseudo-*V* genes *(470)*. The frequency of successful gene conversion occurs every 10–15 cell cycles.

A single *V* gene can undergo 4–10 conversion events in the bursa before the B cells emigrate to peripheral lymphoid organs *(471)*. Recently, it was shown that the activation-induced cytosine deaminase enzyme is required for the gene conversion process *(472)*.

It is not clear how the B-cell repertoire is positively selected in the bursa posthatching. The bursa is a gut-associated lymphoid tissue (GALT) and a site of antigen trapping. Thus, exogenous and gut-derived antigen can be actively transported across the bursa epithelium into bursal lymphoid follicles *(473)*. Arakawa et al. studied the percentage of productive V-J joints in posthatch bursa from normal chickens and from chickens injected into the bursa lumen with an NP-BSA conjugate *(474)*. They found a significant increase in productive V-J joints in the $V_L$ gene after injection of antigen. These results strongly suggest that after hatching, the diversification of the B-cell repertoire in bursa can be shaped after exposure of B cells to environmental antigen.

The process of B-cell repertoire diversification in birds shows that the basic genetic mechanisms have been conserved during the evolution of vertebrates but that the avian evolved different mechanisms governing B-cell repertoire diversification after birth.

## 4. DIVERSIFICATION OF THE MURINE NEONATAL REPERTOIRE

As in all mammalian species, the murine neonatal repertoire results from the random combinatorial association of gene segments (*V[D]*J, $V_LJ_L$) encoding the specificity of the BCR.

The functional $V_H$ gene results from the recombination of 100–500 $V_H$, 12 D, and 4 functional J segments located upstream of *constant region* genes. $V_H$ genes located on chromosome 12 tend to cluster and map contiguously to one another with some interspersions, particularly for those located closest to the $D_H$ cluster *(475)*. Based on nucleotide sequence homology and chromosome position, the $V_H$ genes were classified into 12 $V_H$ gene families *(476)*. The number of $V_H$ genes composing each family varies considerably from one *($V_HX24$)* to several hundred *($V_HJ558$)* related members.

$V_K$ genes located on chromosome 6 result from the combinatorial association of 300 $V_K$ and 4 functional $J_K$ gene segments. Like the $V_H$ genes, based on nucleotide sequence homology, the $V_K$ genes were classified into 20 $V_K$ families; each family contains from 1 to 25 related members *(475,477,478)*. In mice, 95% of immunoglobulins express the κ light chain.

The Vλ locus is the smallest. It is located on chromosome 16 and is composed of four units: Vλ2-Jλ2, VλX-Jλ2,Vλ1-Jλ3, and Vλ1-Jλ1). The *V*λ genes encode 5% of murine immunoglobulins.

Table 16 illustrates the position of $V_H$, $V_K$, and Vλ families on mouse chromosomes 12, 6, and 16, respectively.

Three major methods were used to study the expression of V gene families in fetal, neonatal, and adult B cells. These consisted of Northern blot assay using RNA extracted from cells or hybridomas, single cell *in situ* hybridization, and complementary DNA (cDNA) library analysis.

Study of the utilization of $V_H$ and $V_K$ gene families might shed light on the mechanisms that play a critical role in the generation of the antibody repertoire. In principle, the antibody repertoire is optimized for efficient protection and defense of the host against microbes, whereas the host is protected from danger by elimination of B cells that produce pathogenic autoantibodies.

In adult mice, the $V_H$ gene family usage is random and correlates to the complexity and the size of a given family *(479–481)*. For example, whereas the $V_H$J558 family, which contains more than 50 members, is expressed in 40–50% of B cells, the $V_H$X24 gene family, which contains 1 member, is expressed

**Table 16**
**Position of *VH* and *VK* Gene Families on Heavy and k-Light Chain Loci**

| $V_H$ locus |
|---|
| 5'-J558-3606-$V_H$10 J606-$V_H$12-3609N-VGAM-3660-S107-VGAM-$V_H$11-X24-SM7-S107-Q52-7193-3' |
| $V_K$ locus |
| 5' (VK11-VK24-VK9-VK26)-(Vk1-VK9)-(VK4-VK8-VK10-VK12-VK13-VK19)-(VK28-RN7S)-VK23-VK21 JK 3' |

in 1–2% of B cells *(482)*. It is noteworthy that there are strain variations in the utilization of $V_H$ gene families. For instance, up to a threefold difference has been observed in the case of $V_HJ558$ gene family (most distal) usage vs $V_H7183$ family (most proximal to the $D_H$ gene cluster) usage *(483)*. In sharp contrast, a biased usage of $V_H$ gene families was observed in the early phases of embryonic development, specifically in pre-B cells. Analysis of $V_H$ gene family expression in Abelson murine leukemia virus (MuLV)-transformed pre-B-cell lines *(484)* or fetal liver pre-B-cell hybridomas *(485)* obtained from Balb/c mice showed that the $V_H7283$ gene family and in particular one of its members, $V_H81X$ (located closest to the D locus), was preferentially rearranged. In Abelson MuLV-transformed pre-B-cell lines obtained from the NIH/Swiss outbred strain of mice, the $V_HQ52$ locus, also located proximally to the $D_H$ locus, was highly utilized *(486)*. These data showed that there is a position-dependent expression of $V_H$ gene families biased toward the utilization of proximal $V_H$ gene families during the development of B cells. It is interesting to note that, whereas in pre-B cells the frequency of $V_H81X$ gene rearrangements are functional (>80%), in the adult they are virtually nonfunctional, and this gene is rarely expressed in B cells producing antibodies *(487,488)*.

The expression of the rearranged $V_H81X$ gene as a transgene blocks B-cell development at the $CD43^+$ pre-B-cell stage *(489)*. This can be related either to inhibition of both productive and nonproductive rearrangements of endogenous *V[D]*J genes or to an inability to associate with the Vλ5 surrogate light chain.

In $CD43^+$ pro-B cells, the surrogate λ5 is necessary for the assembly of an Ig-like μ/λ5 transmembrane complex that is necessary for activation of signaling transduction pathways leading to differentiation and proliferation of pro-B and pre-B cells. Studies carried out on transformed pre-B-cell lines showed that two $V_H81X$/μ chains, exhibiting different $V_H$-$D$-$J_H$ joining sequences, do not assemble covalently with a λ5 light chain *(490)*. Therefore, the blockage of B-cell development observed in $V_H81X$ transgenic mice can be related to for-

mation of a $V_H$ μ/λ5 complex required for triggering proliferation and/or differentiation at key checkpoints during the development of B cells. The high utilization of the *$V_H$7183* family observed before the expression of the BCR during the earliest stages of the development of B cells that express a surrogate μ/λ5 receptor indicate that this phenomenon does not result from antigen selection but is a consequence of programmed rearrangement.

A decline of *$V_H$X81* utilization was observed in fetal liver B cells at days 16 and 18 of gestation *(491)*. It is interesting to note that targeted mutation of the *$V_H$81X* gene does not alter the development of the B-cell repertoire in spite of the fact that expression of the *$V_H$7183* gene is affected. This may result from a compensatory effect by utilization of other members of the *$V_H$7183* family, which is composed of 12 different but highly homologous gene segments *(492)*. Alternatively, it may be because of the presence of the $neo^r$ gene used for disruption of the *$V_H$81X* gene. Such a disruption can affect other members of the *$V_H$7183* family located in proximity to *$V_H$81X* or can prevent $V_H$ gene replacement of *$V_H$7183* genes that preferentially use *$V_H$81X* joints *(492)*.

The simplest interpretation of position-dependent biased usage of proximal $V_H$ gene families is $V_H$ gene accessibility to rearrangement. The recombinational machinery works via a one-dimensional "tracking" mechanism that scans upstream from the $DJ_H$ complex for $V_H$ segments located at the 3'end *(493)*.

The neonatal repertoire is not different from the fetal liver repertoire and is characterized by high utilization of the *$V_H$7183* and *$V_H$Q52* gene families *(491,494–496)*. However, Malynn et al. have found high levels of transcripts of the *$V_H$3660* gene family in livers of 1-d-old and in the spleen of 3- and 7-d-old Balb/c and C57BL/6 mice *(495)*. The *$V_H$3660* family is located more distally to the $D_H$ locus between the *VGAM* and *$V_H$S107* families in the center of the murine $V_H$ locus. This suggests that the expression of the $V_H$ gene family in the neonatal repertoire is not strictly position-dependent.

The *$V_H$7183* gene family is composed of 14 different germ line genes *(496)*. Apparently, biased usage among the members of the *$V_H$7183* family exists in neonatal B cells. Thus, the 7183.2 and 7183.6 genes were expressed solely in neonates, whereas the 7183.9 and 7183.11 genes were preferentially expressed in B cells from adult mice *(496)*. This observation suggests that the utilization of different members of the *$V_H$7183* gene family in the neonatal repertoire also is developmentally programmed. This explanation will be strengthened when the position of various members of the *$V_H$7183* gene family are mapped, because it is known that various members of the *$V_H$7183* family are interspersed.

It is probable that neonatal B cells that use *$V_H$7183* produce antibodies that exhibit low-affinity and multispecific binding activity, because it is well known that such antibodies predominate in the neonatal repertoire *(497)*. Indeed, analy-

sis of the binding specificity of monoclonal antibodies produced by hybridomas obtained by fusion of LPS-stimulated cells from Balb/c, New Zealand (NZB), and MRL mice and selected for the expression of $V_H7183$ genes showed that they exhibited multispecific activity. Such antibodies bound to various self-antigen such as myelin basic protein, thyroglobulin, cardiolipin, Sm, mitochondria smooth muscle, and nuclear and glomerular basement membrane antigen *(498)*. However, it should be noted that biased expression of the $V_H7183$ family was observed not only in neonatal B cells producing multispecific antibodies but also in neonatal B cells producing influenza virus HA-specific antibodies *(499)*.

Targeted recombination may play an important role in biased expression of proximally ($V_H7183$, $V_HQ52$) vs distally located ($V_HJ558$) family members. The opportunity for targeted recombination in most D-J combinations is owing to the fact that all the $D_H$ genes have AC nucleotides at the 3'-end, and $J_Hs$ have a TAC near the 5'-end. Targeted recombination using AC overlap will result in a D-J combination that is in-frame *(500)*. Analysis of the 3'-end of the nucleotide sequence of $V_H7183$ and $V_HQ52$ genes showed a sequence that overlaps with the 5'-end of $D_H$ segments. This implies that all rearrangements will be productive and that the final *V[D]*J rearrangements will be in-frame *(484,501)*. Because most neonatal D-J rearrangements have the *D* region in reading frame 1 *(502)*, this should allow preferential expression of $V_H7183$ vs $V_HJ558$ genes in neonatal B cells. This concept is supported by the results obtained in competitive recombination substrate assays demonstrating that the recombinant signal sequence (RSS) for $V_H81X$ is preferred over the consensus for the $V_HJ558$ RSS. These findings suggest that RSS differences may play an important role in biased expression of proximal $V_H$ gene families in neonatal B cells *(503)*.

Study of the structure of *D* and $J_H$ segments expressed in neonatal B cells showed additional differences compared to adult B cells.

In contrast to fetal B cells, in which a relative increase in the utilization of *DQ52* (the most 3' $J_H$ proximal D segment) was noted, in neonatal B cells two different observations were made. In one reported experiment, the preferential utilization of *Dfl16.1*, 5', and *Q52*, the most $J_H$-proximal segments, were highly expressed in immature B cells *(504)*. In 1-d-old mice, a high usage of *Sp2* and *Q52 D* segments was observed *(505)*. These differences may be because of RSS of various *D* segments preferentially targeted recombination of certain *D* segments.

In contrast to fetal B cells in which a distinct preference for $J_H4$ and a relative increase in $J_H1$ usage was observed *(505)*, in 1-d-old mice decreased usage of $J_H4$ and almost identical usage of $J_H1$, $J_H2$, and $J_H3$ were noted *(502)*.

The most striking difference between neonatal and adult B cells consists of the length and extent of diversity of the *CDR3*, which can explain why the neonatal repertoire is more restricted compared to the adult repertoire.

Shorter CDR3 segments characterizing neonatal $V_H$ genes can be related to a lack or paucity of addition of *P* and *N* nucleotides. The *N*-region addition is a template-independent process that is mediated by TdT *(506)*, whereas *P* nucleotide addition is template dependent *(507)*. The role of TdT in template-independent *N*-region diversity was clearly demonstrated by comparing the sequences of *V[D]*J junctions in TdT knockout and TdT+/- mice. In contrast to TdT+/- lymphocytes, the sequences of $V_HS107$ and $V_H81X$ $D_HJ_H$ junctions in lymphocytes from TdT–/– mice did not contain *N* regions *(508)*.

Sequence analysis of *V[D]*J rearrangements showed that the *N*-region in neonatal B cells are almost totally lacking *(494,500,509,510)*. Therefore, lack of *N* region may explain the reduced diversity of the neonatal B-cell repertoire.

A shift in biased usage of $V_H7183$ and $V_HQ52$ gene families leading to normalization of adult-like usage was observed between one to two weeks after birth *(494,495)*.

The mechanisms of normalization are not well understood. Normalization can be the result of changes in genetically programmed recombinational or intercellular constraints affecting the repertoire *(493)*. Alternatively, they can result from cellular selection *(511)*. Among various factors, one may consider negative selection of B cells in the periphery because $V_H81X$ is very rarely used in antibodies or by endogenous or foreign antigen to which young animals are exposed. The role of foreign antigen exposure should be taken into consideration in light of the results of $V_H$ gene usage in adult axenic mice. In these mice, less frequent usage of the $V_HJ558$ family and increased usage of $V_H7183$ were observed at a frequency expected from random usage *(512)*.

However, it can not be excluded that $V_H$ gene replacement also may play a role in normalization of the $V_H$ repertoire, because $V_H$ replacement events involving $V_H7183/V_HQ52$ genes were observed *(513)*.

In adult mice, B1 cells are localized mainly in the peritoneal and pleural cavities, and very few (1–2%) are found in spleen. B1 cells display a very restricted repertoire for some autoantigen and a few microenvironmental antigen because of highly biased expression of V gene families. For example, antibodies specific for phosphatidyl choline (PtC), which is a major component of cell membranes, are encoded by $V_H11\text{-}V_K9$ or $V_H12\text{-}V_K4$ *(448,449)*. The more accepted view is that B1 cells arise from distinct progenitors that are committed to generate B1 cells but not B2 cells *(271)*. The generation of the B1 neonatal repertoire was studied in transgenic mice expressing $V_H12$ and $V_K4$ transgenes. The development of B1 cells that express the transgenes that encode antibodies specific for PtC was studied in newborn livers and spleens in two transgenic lines bearing a low- or high-copy number of transgenes. In the first 120 h of postnatal life, the number of PtC-specific B cells was increased 20-fold compared to nontransgenic mice, doubling about every 24–30 h and

becoming predominant at 6 d after birth. These mice do not have PtC-binding B2 cells in spite of the fact that 30% of total B cells are $CD5^-$, namely, B2 cells *(514)*. This observation suggests that $V_H12$ and $V_K4$ can rearrange only in B1 cells, or if this rearrangement also occurs in B2 cells, the B2 cells cannot express a BCR encoded by two *V* genes. This is in agreement with other observations suggesting that B1 and B2 cells use different $V_H$ genes *(506)*. Antigen-driven selection mechanisms may be responsible for restricted usage of *V* gene families in neonatal B1 cells because selection proceeds rapidly after birth. Lack of B cells expressing the transgenes in adult bone marrow indicates that the selection occurs in the periphery and is initiated after Ig gene rearrangement and the expression of the BCR. It is noteworthy that the majority of B cells expressing $V_H12$ are excluded from the peripheral repertoire in adults, with the exception of cells expressing a CDR3 composed of 10 amino acids including a glycine in position 4 (designated as 10/G4). Thus, in contrast to the neonatal repertoire, the adult $V_H12$ is restricted by clonal expansion of a minute subset that has a particular CDR3 and binds PtC.

Taken together, these findings show that there are important differences between the usage of $V_H$ genes in neonatal B1 and B2 cells and between neonatal and adult B2 cells.

In mice, the $V_K$ light-chain genes contribute significantly to antibody diversity because 95% of murine Ig molecules bear a κ chain. In adult mice, all $V_K$ gene families are expressed without evident biased usage of a given family. Study of $V_K$ gene family expression in neonatal B cells showed, similarly to adult mice, that all families are expressed *(515,516)*. It is particularly important to note that usage of the $V_K21$ family, which is located the most proximal to $J_K$, is not increased in the fetal *(515)* and neonatal *(516)* repertoires. This finding demonstrates that $V_K$ gene expression does not follow the $V_H$ paradigm, which consists of the skewed $V_H$ gene family expression in the neonatal repertoire.

Thus, if a position-related process is involved in the expression of $V_H$ gene families in pre-B- and neonatal-cell repertoire until 7 to 14 d after birth, then a similar mechanism is not operating at the $V_K$ locus.

Table 17 shows the complexity and the expression of the $V_K$ family in newborn and adult LPS-stimulated B cells. An important factor in the generation of antibody diversity consists of the pairing of $V_H$ with $V_L$ genes. For example, the random association of $10^4$ $V_H$ and $V_K$ would give $10^8$ different antigen-binding sites. The analysis of the pairing of $V_H$ with $V_K$ genes in polyclonally activated B cells shows that the pairing is stochastic, with a few exceptions: $V_K1$ and $V_K8$ with $V_HQ52$, *S107*, and *X24* in adult mice and $V_K1$ with $V_HQ52$ in neonatal B cells *(517)*.

**Table 17**
**Expression of $V_K$ Gene Families Among LPS-Stimulated Splenocyte Colonies From Neonatal and Adult C57BL/6 Mice**

| Gene family | *VK2* | *VK22* | *VK24* | *VK1* | *VK9* | *VK4* | *VK9* | *VK10* | *VK19* | *VK21* |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene complexity | 5 | 7 | 2 | 3 | 11 | 8 | 12 | 5 | 10 | 10 |
| $V_K$ expression in | | | | | | | | | | |
| Neonatal | 4.7[a] | 0.2 | 0 | 40.1 | 23 | 5.4 | 13.8 | 0.3 | 1.2 | 0 |
| Adult | 1.7 | 4.3 | 0.3 | 25.8 | 5.1 | 3.3 | 9.0 | 1.4 | 6.1 | 2.6 |

[a]Percentage of total $C_K^+$ colonies.
Neonatal mice were 4 to 6 d old and adult mice were 14 to 24 wk old.

Besides somatic recombination and pairing of various *V* gene segments and *N*-addition, two other processes, somatic hypermutation and receptor editing, play an important role in diversification of the repertoire.

In *V* genes, somatic hypermutation generates mutations at a rate six orders of magnitude higher than the rate of spontaneous mutations in other mammalian structural genes *(518)*. One of most interesting aspects of the process of somatic mutation in *Ig* genes is its targeting to rearranged heavy- and light-chain *V* genes, whereas the genes encoding the constant region of heavy and light chains are stable.

The majority of somatic mutations in *V* genes are base pair mutations resulting in replacement or silent mutations *(518)*. Small insertions or deletions were observed only rarely *(519)*. Point mutations begin to appear near the *V* gene at a frequency of $10^{-2}$ mutations/bp/cell generation *(520)*, and then in *V* genes at a frequency of $10^{-3}$ to $10^{-4}$ mutations/bp/cell generation *(521,522)*. Somatic mutations are antigen-dependent events that arise during the immune responses against T-cell-dependent antigen that occur in germinal centers (GCs). Somatic mutations in Ig genes contribute to the increased affinity of antibodies and to better and more efficient defense reactions.

Antibody responses against T-cell-dependent antigen can be induced in neonatal life. Structural analysis of *V* genes expressed in antibodies specific for (T,G)A-L polymer and NP-CGG conjugate showed that somatic mutation, memory generation, and repertoire shift occurred subsequent to priming of neonates with these antigen *(523)*. Primary antibody responses to synthetic polymers are restricted to side chain T and G and, in A/J mice, are dominated by B cells expressing the $V_H$, *H10* gene, which is from the small $V_H$*SM7* family, and the $V_K1$ light chain. Like adults, neonates immunized with (T,G)A-L make a primary response to side-chain amino acids and to the A-L epitope only when immunized with synthetic polymer coupled to methylated BSA in Freund's complete adjuvant (FCA) at age 5 to 7 d *(523)*. Sequence analysis of 48 neonatally rearranged $V_H$*H10* genes showed that 34% of $V_H$*H10* gene had 1, 17% had 2, 10% had 3, and 2% had 6 bp nucleotide changes. A closed rate of mutation was observed in the $V_H$ genes from mice immunized with NP-CGG conjugate at d 1 to 2 after birth and analyzed 14 to 28 d after priming *(523)*.

These elegant studies demonstrated that in neonatal mouse B cells, the DNA replicative machinery, a prerequisite for the induction of somatic mutations, is active within 1 d of birth.

BCR engagement may lead either to elimination of autoreactive cells during B-cell development or to increased diversity in peripheral B cells by a receptor editing process. The process leading to elimination of autoreactive cells takes place in pre-B cells and is called "receptor editing." In peripheral B cells, the

process contributing to increased diversity of the repertoire is called "receptor revision" to distinguish it from receptor editing leading to tolerance.

Both processes consist of the capacity of $V_L$ or $V_H$ to undergo successive rearrangements. The receptor editing process was explored in several strains of transgenic or knockin mice. The first detailed demonstration of $V_L$ chain editing was provided by a study of anti-DNA heavy-chain transgenic mice in which it was shown that a receptor editing of the double-stranded DNA (dsDNA)-specific BCR. This resulted from the secondary rearrangements of $V_K$-$J_K$ *(524)* that reduced the diversity of the observed $V_K$ repertoire in the periphery and biased usage of $J_K5$ segment *(525)*. Studies of four separate *Ig* knockin mouse strains showed that about 25% of light chains expressed in the BCR of developing B cells in vivo were produced by receptor editing *(526)*. Magari et al., using a knockin mouse strain expressing a rearranged *V[D]*J gene, showed that light-chain rearrangement in peripheral B cells contributed to the generation of high-affinity antibodies *(527)*. This finding strongly suggested that in addition to the somatic hypermutation process, the receptor revision process can contribute to the diversification of the repertoire. For receptor editing or receptor revision processes to operate, a signaling of the BCR expressed in pro-B and pre-B cells is required to retain active recombination machinery, namely RAG enzymes. The fact that *RAG* genes are expressed in pro-B and pre-BII cells explains the high frequency of receptor editing in these cells *(526)*. The re-expression of *RAG* genes is required in neonatal or adult B cells for a secondary rearrangement to occur. The possibility of the re-expression of *RAG* genes in mouse peripheral B cells was demonstrated in activated mature GC B cells *(528)* occurring after antigen stimulation *(529–531)*.To investigate possible receptor revision in newborn mice, we studied expression of the *RAG2* gene in 7-d-old mice immunized with an empty plasmid as control (pC) or with a plasmid containing the *HA* gene of the WSN strain of the influenza virus (pHA). Figure 28 shows the occurrence of GCs 7 d after immunization, which disappeared by 28 d as assessed by staining of spleen sections with anti-PNA antibodies. Centrocytes were detected in GCs 7 to 28 d after immunization with monoclonal anti-GL7 antibody, and their presence paralleled the detection of *RAG1* activity in GCs as assessed by staining with anti-*RAG1* monoclonal antibody. The absence of $GL7^+$ and $RAG1^+$ cells in the spleen of a mouse immunized with empty plasmid indicated that the induction of *RAG1* activity did not result from CpG motifs in empty plasmid but from the viral peptide encoded by the gene expressed in pHA plasmid. The data presented in Table 18 show a significant increase of $B200^+$ $GL7^+$ cells in the spleen of mice 7–21 d after immunization with pHA but not in mice immunized with control plasmid. Figure 29 demonstrates that the expression of *RAG1* was limited to sorted $B220^+$

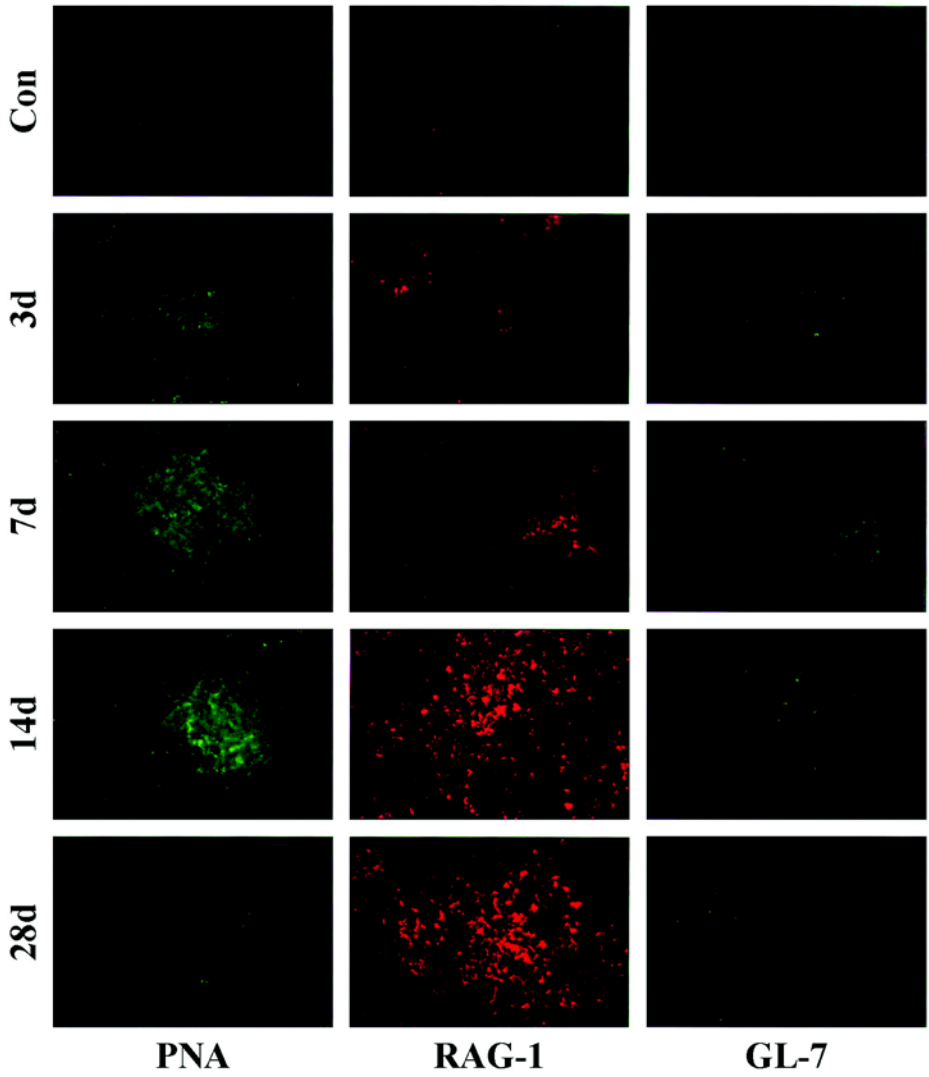


Fig. 28. Expression of RAG-1 protein in splenic germinal centers of 7-d-old Balb immunized with a empty plasmid (pC) and a plasmid-counting influenza virus hemagglutinin gene (pHA). Spleen specimens from 1-d-old mice immunized with empty plasmid or with pHA were harvested 3, 7, 14, and 28 d after immunization. The spleens were embedded in 10 × 10 mm cryomolds (Fisher Scientific, Springfield, NJ) using Histp Prep. Serial 7 µm sections were fixed for 10 m at –20 C in acetone, methanol (1:1) and stained with 10 µg/mL FITC-anti-PNA (left panel), R-PE anti-RAG-1 (center panel), or FITC anti-GL-7 monoclonal antibodies. After extensive washings with 1% BSA in PBS, the sections were coverslipped with Vechtashield and examined in a Zeiss Axinphot microscope (×100).

**Table 18**
**Percentage of B220+, GL7+, B220+GL7+ Cells in Spleen After Injection with Empty Plasmid (pC) or a Plasmid Containing Hemagglutinin of WSN Infuenza Virus Strain pHA**

| Day after injection | pC | | | pHA | | |
|---|---|---|---|---|---|---|
| | B220+ | GL7+ | B220+GL7+ | B220+ | GL7+ | B220+GL7+ |
| 3 | 53.3[a] | 0.2 | 0.9 | 61.2 | 0.2 | 0.9 |
| 7 | 47.8 | 0.6 | 1.4 | 53.3 | 7.1 | 6.4 |
| 14 | 44.5 | 1.5 | 1.7 | 47.5 | 9.5 | 16.5 |
| 21 | 41.6 | 1.5 | 1.3 | 48.1 | 2.8 | 5.2 |

[a]Percentage of B220+, GL7+, B220+GL7+ cells was analyzed in lymphocyte population gate.

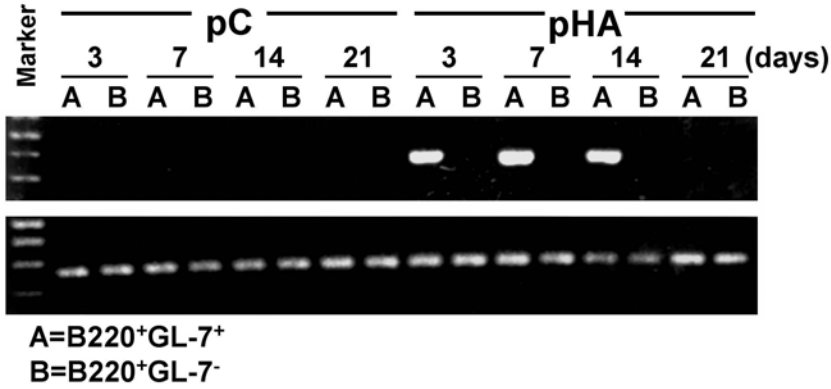


Fig. 29. Detection of RAG-2 transcripts in spleens of 7-d-old Balb/c mice immunized with pC and pHA plasmids. Single cell suspension of B200$^+$GL-7$^+$ and B220$^+$ GL-7$^-$ cells sorted from spleens of animals harvested d 3, 7, 14, and 21 after immunization were used to extract cellular RNA. 8 µl of total RNA obtained from $5 \times 10^6$ sorted cells was treated with DNaseI and was reverse transcribed with primers specific for RAG-2 and β-actin. PCR products were separated onto 1% agarose gel and visualized with ethidium bromide. pC or pHA stand for RNA extracted from spleens of 7-d-old Balb/c mice immunized i.m. with 100 µg of pC or pHA. 3, 7, 14, and 21 stand for days of harvesting the spleen after injection of plasmids. A, RNA extracted from BB220$^+$GL-7$^+$ sorted cells; B, RNA extracted from B220$^+$GL-7- sorted cells.

GL7$^+$ B cells in spleens harvested 3,7, and 14 d after immunization with pHA plasmid but not in B200$^+$ GL7$^-$ B cells. (Data not published.)

Taken together, these results demonstrated that GCs are induced by the immunization of 7-d-old mice with a plasmid containing a viral gene. The occurrence of a GC is associated with an increased number of B cells expressing the GL7 marker. The RAG1 transcript was detected in B200$^+$ GL7$^+$ before the

occurrence of organized GCs and lasted for 14 d, whereas the RAG1 protein was detected 7 d after immunization and lasted until d 28. These findings strongly suggest that the immunization of neonates with naked DNA induced the expression of RAG1, which, via secondary rearrangements, can contribute to the diversification of the neonatal repertoire.

## 5. DIVERSIFICATION OF THE B-CELL REPERTOIRE IN RABBITS

In rabbits, the IgH locus, as in other mammalian species, consists of multiple $V_H$, *D*, and $J_H$ segments. It contains a minimum of 100 $V_H$ genes separated from each other by 5 kb. Among approx 100 $V_H$ genes, 50% are functional. Because they exhibit high nucleotide homology, they are considered members of the same $V_H$ gene family. The $V_H1$ gene is the most proximally located. It appears that, among a large variety of $V_H$ genes, only four are used in *V[D]*J rearrangements, and $V_H1$ is used in about 80% of functional rearrangements.

The *D* gene locus contains 11 segments spanning over 20 kb of DNA, and there are 5 functional $J_H$ segments *(532)*. The constant heavy-chain locus contains 16 $C_H$ genes: *C*μ, *C*γ, *C*ε, and 13 *C*α genes. Like mice, in rabbits, 80–90% of *Ig* express the κ light chain. The κ locus in rabbits is more complex than in mice because it contains two distinct *C*κ genes: *C*κ1 and *C*κ2. Each *C*κ gene is associated with one cluster of *J*κ and two clusters of *V*κ genes. The rabbit λ locus also is more complex than in mice and more closely resembles the human λ locus, containing four *V*λ genes and as many as eight *C*λ genes (reviewed in ref. *272*).

B lymphopoiesis in rabbits begins at days 17–19 of gestation, when pre-B cells can be detected in fetal liver. B-cell lymphopoiesis late in fetal life and at the first day after birth switches from the liver to bone marrow. Most B cells that develop early in ontogeny migrate soon after birth to the GALT system, which in rabbits is composed of appendix, *sacculus rotundus*, and Peyer's patches. After birth, in rabbits, few B cells develop because they exhibit a long half-life. Somatic diversification of the B-cell repertoire occurs in the GALT system. Exposure of B cells from the GALT system to bacteria may play a role in the diversification of the repertoire because the GALT system in germ-free rabbits is poorly developed, and these rabbits are highly immunocompromised *(533)*.

There are some similarities and differences between the molecular pattern of development of murine and rabbit fetal and neonatal repertoires.

First, *V[D]*J rearrangements were detected in 14-d-old fetuses, and $V_H1$ (the most proximal $V_H$ gene) was found preferentially rearranged in 14-to 28-d-old fetuses *(534)*. In newborn and 1-wk-old rabbits, $V_H1$ was found in 80% of *V[D]*J rearrangements *(535)*. This indicated that, similarly to mice, there are position-dependent rearrangements of $V_H$ genes. High usage of $V_H1$ in the neo-

nates may explain the restricted rabbit neonatal repertoire and minimal antibody response to various antigen, even in 2- to 3-wk-old rabbits *(536,537)*.

In contrast to mice, in which the D-J segments are first rearranged in pro-B cells, in rabbits, the $V_H$-$D_H$ rearrangement is a more frequent initial rearrangement among $V_H$ gene segments *(538)*. Also, whereas in mice DQ52 (the 5'-most $D_H$ gene) is preferentially expressed in the neonatal B-cell repertoire, the rabbit *DQ52* gene was not used in *V[D]*J rearrangements in 19- to 28-d-old fetuses or in the bone marrow from a 28-d-old fetus. This may result from the fact that, in rabbits, the 3'-RSS of *DQ52* shows an atypical nonamer. The *Df* gene located in the center of the $D_H$ locus was preferentially used in *V[D]*J rearrangements from 15- to 28-d-old fetuses *(539)*.

In rabbits, the *V[D]*J genes do not diversify until after birth. Actually, Short et al. found that in 3-wk-old rabbits, the majority of *V[D]*J genes were not diversified, whereas at 9 wk of age they were highly diversified *(540)*. This diversification resulted from *N*-addition and to a high frequency of gene conversion in $V_H$ genes. Gene conversion was observed in both the CDR and the framework segments *(532)*. Although the *N*-addition was observed starting at d 14 of gestation *(272)*, the diversification by gene conversion occurs later during the first few weeks of life mainly in the GALT system, namely, in the appendix of 6-wk-old rabbits *(541)*.

In summary, these findings show that there are some particular molecular features that contribute to the establishment of the B-cell repertoire in rabbits. First, neonatal B cells that developed during ontogeny have a long half-life, and very few B cells develop in adults. Second, the restricted neonatal repertoire is related to biased usage of the $V_H1$ and *Df* segments localized to the center of the $D_H$ locus. Third, diversification of the repertoire resulting from *N*-nucleotide addition occurs in embryonic life, whereas the diversity resulting from gene conversion occurs later in postnatal life, mainly in the GALT system.

## 6. DEVELOPMENT OF THE B-CELL REPERTOIRE IN SHEEP AND CATTLE

In sheep, the majority of immunoglobulins bear the λ light chain. The sheep *Ig* variable repertoire consists of nine different $V_H$ genes, six of which are functional. Based on the high sequence homology of functional $V_H$ genes (>80), it was considered that all six $V_H$ genes belong to a single $V_H$ gene family. The $V_H$ segments combine with a large set of very heterogeneous *D* segments and with a small number of $J_H$ segments *(542)*. In sharp contrast, the λ locus is composed of greater than 100 germ line genes that recombine with only two Jλ segments, *J*λ1 and *J*λ2, among which *J*λ1 is more frequently used than *J*λ2 *(543)*. Among numerous $V_L$ genes, only a few (20–30) were used and found to be rearranged.

Six different $V_K$ genes were identified that, based on sequence homology, were classified into four $V_K$ families. *$V_K$3* and *$V_K$4* families each contain a single member. The $V_K$ genes recombine with two $J_K$ segments.

The most fascinating aspect of the development of the sheep B-cell repertoire consists of two distinct features. First, the establishment of the repertoire in fetal lambs is completely independent of antigen exposure because ovine placenta is impermeable to macromolecules. Second, mature $IgM^+$ B cells appear early in ontogeny, around d 45 of gestation. They migrate and colonize the spleen at d 40 of gestation and then the ileal Peyer's patches, where the B-cell repertoire is rapidly expanded at 70–100 d of gestation. Third, the B-cell compartment matures during fetal development, as demonstrated by the ability of fetal lambs to produce antibodies after immunization with various antigen. Fourth, structural analysis of sheep *V* genes demonstrated that no new rearrangements occur after the initial colonization of the ileal Peyer's patches during the second half of fetal life *(545)*. Although there are no data on the structure of $V_H$ genes expressed in fetal lambs, analysis of a genomic DNA library from an adult sheep showed that rearranged *V[D]*J exhibits large nucleotide variation in *CDR1* and *2*, extensive *N*-nucleotide addition, and D segments of various lengths and sequences *(542)*. Thus, the $V_H$ diversity results from somatic mutation rather than combinatorial mechanisms. In addition, no indication of gene conversion was noted. In early stages of embryonic life (61- to 90-d-old fetuses), all $V_K$ family members were more or less equally represented. Meanwhile, in older fetuses, a biased usage was observed because nearly all $V_K$-$J_K$ rearranged genes exclusively used *$V_K$3, $V_K$4*, and *$J_K$2*. Sequences of *$V_K$3* and *$V_K$4* showed little variation in either CDRs or framework segments *(546)*.

Diversification of the *V*λ repertoire results from combinatorial rearrangements of multiple functional *V*λ genes with a marked absence of *P*- or *N*-nucleotide addition *(547)*, as well as from antigen-independent hypermutation processes *(548)*. Comparison of the sequences originating from fetal or 1-d-old lambs to those of adult animals indicates little diversification in young lambs. The striking nucleotide differences between the neonatal and adult *V*λ repertoires strongly suggest that they result from accumulation of mutations with age *(547)*. Taken together, these findings show that the diversity in the fetal and neonatal repertoires is the result of limited $V_H$ and $V_K$ usage to few mutations and to the absence of *P*- and *N*-nucleotide addition in $V_L$ genes. The diversification process takes place mainly in ileal Peyer's patches that may represent the equivalent of the Bursa of Fabricius in avians.

In cattle, the primary source of B-cell progenitors is the fetal liver and spleen. No B cells were found in the bone marrow in the third trimester of gestation or in 2- or 12-d-old calves *(549)*. After birth, the spleen is likely to be the organ of

gene rearrangement because *RAG1* gene expression was found in the spleen of 14-d-old calves and disappeared in 32-wk-old cattle *(550)*. Similarly to sheep, in cattle, the B cells might migrate to ileal Peyer's patches where they undergo proliferation and the diversification of *Ig* genes.

Restriction fragment length polymorphism (RFLP) analysis of bovine genomic DNA indicated the $V_H$ locus contains many $V_H$ genes. However, comparison of germ line gene sequences with the $V_H$ sequences from adult splenic cDNA indicated that the B-cell repertoire is dominated by a single $V_H$ family comprised of as few as 15 members and closely related to the human $V_H2$ or murine *Q52* $V_H$ families. The *D* segments are of greater length, and at least two $J_H$ segments are used *(551,552)*.

The majority of bovine Ig bears a λ light chain. The λ locus contains 20 *V*λ germ line genes. Among them are 14 pseudogenes that exhibit various structural defects, including a lack of RSS at the 3'-end, stop codons, truncations, insertions, or deletions resulting in the loss of reading frames. Two *J*λ segments were identified in genomic DNA, but all the expressed *V*λ genes examined contained a single *J*λ segment. The *V*λ-*J*λ can associate with four different *C*λ genes *(553)*. Apparently, in cattle, the $V_K$ locus is complex but not well studied.

In fetal livers, only the transcripts of the bovine $V_H1$ family were detected *(551)*. The diversity of the $V_H$ probably lies in the length of *CDR3*, which ranges from 13 to 28 codons. The putative *D* genes are read in one reading frame. One $J_H$ segment seems to be used throughout life, because it was found in cDNA sequences of $V_H$ genes from both fetal and adult cattle. The diversification of the *V*λ repertoire was studied by comparing the complete *V*λ cDNA sequences obtained from ileal Peyer's patches from 11-d-old and 32-wk-old calves. This analysis demonstrated that diversification had occurred by 11 d after birth, when the majority of rearranged *V*λ genes used the *V*λ B4 gene family. This gene usage was found in the majority of cDNA sequences of older animals, indicating that *V*λ genes expressed in the neonatal repertoire are maintained in adult life. Structural differences were clustered in CDR segments with a few scattered changes in framework regions. The same V-J junction was observed in all clones analyzed, indicating that rearranged *V*λ gene segments recombined by a single predominant rearrangement *(553)*. These observations suggest that combinatorial mechanisms are unlikely to be significant in the generation of antibody diversity in calves. In contrast, there are several findings strongly suggesting that gene conversion soon after birth is the major mechanism leading to antibody diversity. The major argument is that the variation observed in rearranged *V* genes compared to germ line genes showed that there are clusters of nucleotide changes and that they can originate from the *V*λ pseudogenes, which function as sequence donors.

In ileal Peyer's patches, the fact that the *Vλ* genes already exhibit sequence variation suggests that significant diversification of the repertoire occurs in fetal spleen and within a few days after birth in the GALT system.

## 7. DEVELOPMENT OF THE HUMAN NEONATAL REPERTOIRE

In humans, 60% of immunoglobulins bear a κ light chain, and 40% bear a λ light chain.

The $V_H$ locus located on chromosome 14 represents a region of 957 kb of DNA containing 123 $V_H$ genes, among which 39 are functional, 29 are pseudogenes, and others have non-rearranged open reading frames *(554)*. Based on >80% DNA homology, the $V_H$ genes are grouped into seven families. The $D_H$ locus consists of 9.5-kb tandem repeat units that contain 29 germ line genes; of these 25 are functional and are classified into 6 $D_H$ families. There are nine $J_H$ segments, of which six are functional.

The $V_K$ locus located on chromosome 2 contains 76 germ line genes; of these, 32 are functional. They are organized into two cassettes: the first, composed of 40 genes, is located in the $J_K$ proximal region; the second, composed of 36 genes, is located in the distal inverted region. The $V_K$ genes were classified into seven families or subgroups. All five $J_K$ segments are functional *(555,556)*. $V_H$ and $V_K$ genes may be found at other chromosomal locations where they are not expressed. The loci containing these nonexpressed genes are called orphons. It is believed that orphons arise by inversions and conversions that have been maintained during evolution. A $V_H$ orphon was identified on chromosome 16, and a $V_K$ orphon was found located close to the centromere on chromosome 2.

The *Vλ* locus located on chromosome 22 is composed of 70 *Vλ* genes, of which 30 are functional, and 7 *Jλ* segments, of which 4 are functional *(557,558)*.

Generation of the neonatal repertoire results from random combinatorial events of the segments encoding $V_H$, $V_K$, and *Vλ* genes.

The rearrangements of *V* genes in humans during embryonic life begin at 7–8 wk of gestation in the fetal liver when the first wave of pre-B cells is detected. The pre-B cells are characterized by the presence of cytoplasmic *IgM* and a *Vλ*-like chain and lack of surface *IgM*. The pseudo-light-chain cluster contains three genes (*14.1, Fl1,* and *16.1*) located on chromosome 22 close to the *Vλ* locus. Structural analysis of the pseudo-λ genes shows similar organization and high nucleotide homology, suggesting that the two systems,(λ and λ-like, diverged after duplication of a common ancestor *(559)*. In fetal livers (8 wk of gestation), only transcripts of the *16.1* λ-like gene were detected in the large cDNA library analyzed *(559)*.

Immature B cells ($sIgM^+$), followed by mature B lymphocytes ($sIgM^+$, $sIgD^+$), were detected at 7–8 and 12 wk of gestation, respectively. During

intrauterine development, Ig class switching occurs only in a few B cells, indicating that this process is independent of antigen exposure because the intrauterine environment is sterile.

Analysis of the expression of sIg and CD5 showed that 88% of neonatal $CD19^+$ B cells express sIgD, and about 80% expressed both IgD and IgM. Only a few (<10%) are $IgM^+IgD^-$. About 50% express CD5 and sIgD and may be considered B1 cells.

Like mice, the rearrangement of V gene segments follows a similar genetic program of rearrangements:

$$D_H \rightarrow J_H,\ V_H \rightarrow DJ_H\ C\mu \text{ and } V_L\text{-}J_L$$

A study of V[D]J rearrangements in human fetal liver at 7, 13, and 18 wk of gestation showed a preferential usage of the *VH3*, *DQ52*, and *Dxp* gene families from 30 estimated D segments and $J_H3$ and $J_H4$. The diversity of CDR3 in fetal liver B cells is limited by the absence of *P* junctions and fewer *N* additions in D-J junctions. Meanwhile, the $D_H$ reading frame appears to be randomly used. The rearrangements of D elements occur by inversion and D-D fusion that could result from unequal crossing over *(560)*. In contrast, other investigators have found preferential expression of $V_H5$ and $V_H6$ in 7-wk-old fetal livers, suggesting a position-biased usage because the *$V_H6$* gene is the most $J_H$ proximally located *(561)*. A biased usage of $V_H6$ also was found in fetal livers from 16- to 24-d-old fetuses *(562)*. Sequence analysis of heavy-chain transcripts obtained from fetal livers at 104 and 130 d of gestation identified by hybridization with a Cμ probe showed the same pattern of usage of *$V_H$* gene segments, because three highly conserved $V_H3$, DQ52, and $J_H3$ and $J_H4$ genes were highly expressed, whereas transcripts from $V_H1$, $V_H2$, $V_H4$, $V_H5$, and $V_H6$ genes were expressed at a low frequency *(563)*. The preferential use of $D_HQ52$ in sterile and mature V[D]J transcripts indicates that nonrandom usage of some gene segments in fetal B cells reflects a genetic regulatory program intrinsic to B cells in this stage of development and that the $V_H3$ genes play an important biological role *(564)*.

Study of the expression of $V_H$ genes in the neonatal repertoire, particularly in cord blood lymphocytes, showed that all $V_H$, $D_H$ families and $J_H$ segments are expressed, with a higher frequency of utilization of the $V_H3$ and $V_H1$; $D_H2$, $D_H3$, and $D_H6$ families; and $J_H6$, $J_H4$, and $J_H5$. A shift in the utilization of $J_H$ segments is evident, because in fetal livers, $J_H3$ was frequently used. Another study reported the frequent use of the $V_H7$ gene family in cord blood B cells *(565)*.

It is known that B1 $CD5^+$ cells predominate in cord blood. Analysis of $V_H$ gene family expression in cord blood-derived Epstein–Barr virus (EBV)-transformed B-cell lines showed frequent usage (30%) of the $V_H4$ gene family. It was shown that the $V_H4$ gene family encodes for self-reactive and multispecific antibodies *(566)*.

One may ask whether the shift in use of $V_H$ gene segments during transition from the fetal to neonatal repertoire is because of exposure to antigen after birth. This question was addressed by studying the expression of $V_H$ genes in preterm neonates (gestational age: 25–29 wk) vs full-term neonates. This study showed that the exposure of preterm neonates to antigen resulted in a higher frequency of class switching to IgG and a higher number of somatic mutations within 2 wk of postnatal life. In contrast, premature exposure to extrauterine environmental antigen did not alter the pattern of $V_H$ expression and did not lead to a switch to an adult-like repertoire. In addition, the length of CDR3 was not increased by *N*-nucleotide addition. This observation clearly indicates that $V_H$ region diversity is mainly developmentally regulated and that the repertoire restrictions persist in spite of premature exposure to antigen *(567)*.

Sequence analysis of the $V_H6$ gene from cord blood B cells showed no mutations; however, almost all sequences from 10-d-old newborns with acute infections had mutations *(568)*. The $V_H6$ gene of 2- and 10-mo-old healthy children exhibited mutations, and the frequency of mutation increased with age *(569)*. Comparison of the $V_H6$ gene sequence expressed in newborns and young children with the $V_H4$ germ line gene showed a mutation frequency of 4.3% with a range from 1 to 24 mutations in the 241-bp gene segment analyzed. The mutation frequency was higher in the CDR (1.6%) compared to the framework region (0.9%) with a replacement:silent ratio of 4 for CDR and 2.9 for framework segments. Comparison of CDR3 length in different infant age groups did not show a significant difference *(569)*. These results show that mutation in the $V_H6$ gene is a rare event in newborns and children younger than age 6 mo and that the frequency of mutation increased after age 6 mo, indicating an antigen-driven process. This explanation is supported by the paralleled increased of the frequency of mutations and the replacement:silent mutation ratios.

Study of the neonatal κ repertoire was carried out using cDNA obtained by unbiased polymerase chain reaction (PCR) amplification of nonproductive and productive rearrangement of $V_K$ genes. Initial studies suggested that the $V_K$ gene family is nonstochastically used, because genes belonging to the $V_K1$ and $V_K3$ families located in the distal half of the locus were predominantly expressed *(570,571)*. A finer analysis was carried out using a single-cell PCR technique, purified $sIgD^+$ $CD5^-$, and $CD5^+$ $sIgD^+$ B cells purified from cord blood *(572)*.

In this study, the expression of $V_K$ families was analyzed in productive and nonproductive rearrangements, which affords the opportunity to understand the molecular mechanisms before the repertoire is shaped by positive and negative selection. This study demonstrated that six out of seven $V_K$ families were rearranged in the neonatal repertoire. In the nonproductive repertoire, the members of $V_K1$, $V_K3$, and $V_K6$ occurred as frequently as expected from genome complexity, whereas the occurrence of $V_K2$ was less frequent than expected. $V_K4$

and $V_K5$ were more frequently used than expected from random use in the productive repertoire. A member of the $V_K3$ family gene, A27, was overrepresented.

Roughly the same pattern of $V_K$ gene family expression was observed in the productive repertoire: $V_K1$ and $V_K5$ were expressed significantly more often in neonatal than adult repertoires. *$V_K3$, $V_K4$, $V_K6$*, and *$V_K7$* gene expression did not differ between the neonatal and adult repertoires, and $V_K2$ was less frequently expressed. Thus, $V_K$ genes from both the proximal and distal cassettes were found in the neonatal repertoire, but those from the distal cassette were found less frequently. With respect to individual $V_K$ genes, genes *B2, L9*, and *O12/O2* were expressed more significantly, accounting for 79% of productive rearrangements in the neonatal repertoire.

Compared to the adult repertoire, the junctional diversity was less marked (28% neonatal vs 57% adult productive rearrangements contained *N*-nucleotide), whereas CDR3 length was comparable (average: 27 nucleotides). In neonatal B cells, $J_K2$ was predominantly used, $J_K5$ was used at the expected frequency, $J_K1$ was used less, and $J_K3$ and $J_K4$ were significantly less used than expected. Seventeen mutations were found in all neonatal $V_K$ rearrangements, indicating an overall mutational frequency rate of $3.27 \times 10^4$ per base pair.

In contrast to $CD5^-IgD^+$ B cells, $CD5^+$ B1 cells showed some important differences. Only productive rearrangement of $V_K$ genes from the distal cassette was found. Meanwhile, no differences in $J_K$ usage and *N*-nucleotide insertions were observed in $CD5^+$ B1 cells compared to neonatal $IgD^+CD5^-$ B2 cells.

These results demonstrated that there is a molecular preference, based on chromosomal position, in the selection of $V_K$ gene families and $J_K$ segments in the neonatal repertoire, leading to a more restricted and uniform repertoire in neonates compared to adults. Limited variability in the generation of $V_K$-$J_K$ joints, less receptor editing, and the shorter length of CDR3 also are important factors that contribute to a more restricted $V_K$ repertoire in neonates. Higher expression of some individual members of the proximal $V_K$ gene families suggests positive selection by exposure to self-antigen rather than to foreign antigen because the intrauterine environment is sterile. High usage of the distal cassette in neonatal B1 cells suggests positive selection by self-antigen, which explains why these cells produce multispecific and self-reactive autoantibodies.

There are no extensive studies of the *Vλ* neonatal repertoire, which in humans represents 40% of immunoglobulins.

A study of *VλJλ* rearrangements in $IgM^+$ B cells from fetal spleens at 18 wk of gestation showed an overrepresentation of the distal *Jλ* cluster recombining with the *Jλ7* segment. *Vλ* genes paired stochastically with $V_H$ genes in fetal spleen $IgM^+$ B cells *(573)*.

In conclusion, the major molecular characteristic of the human neonatal repertoire consists of preferential usage of $V_H$ and $V_K$ families located in the prox-

imity of the J clusters. The restricted and more uniform repertoire is related to less frequent *P* and *N* addition and shorter CDR3 length. The frequency of mutation is considerably lower than in adults because B cells are not exposed to antigen during gestation.