Evidence for V_H Gene Replacement in Human Fetal B Cells

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ABSTRACT

Background: In contrast to evidences of Ig H chain receptor editing in transformed cell lines and transgenic mouse models, there has been no direct evidence that this phenomenon occurs in human developing B cells. **Methods:** V_HDJ_H rearrangements were obtained from genomic DNA of individual IgM^- B cells from liver and IgM^+B cells from bone marrow of 18 wk of gestation human fetus by PCR amplification and direct sequencing. **Results:** We found three examples of H chain receptor editing from IgM^+ and IgM^-human fetal B cells. Two types of V_H replacements were identified. The first involved V_H hybrid formation, in which part of a V_H gene from the initial VDJ rearrangement is replaced by part of an upstream V_H gene at the site of cryptic RSS. The second involved a gene conversion like replacement of CDR2, in which another V_H gene donated a portion of its CDR2 sequence to the initial VDJ rearrangement. **Conclusion:** These data provide evidence of receptor editing at the H chain loci in developing human B cells, and also the first evidence of a gene conversion event in human Ig genes. **(Immune Network 2002;2(2):79-85)**

Key Words: Human, fetal, B lymphocyte, heavy chain, receptor editing

Introduction

Self-tolerance is induced either by deleting or functionally inactivating the autoreactive B cells, or by changing their receptor specificity. One mechanism for changing receptor specificity involves the activity of the products of RAGs, leading to the replacement of the autoreactive BCR by a secondary Ig gene rearrangement, referred to as "receptor editing" (1,2). Receptor editing was initially thought be a characteristic property of the L chain (1,2), since, during initial rearrangement of the H chain locus, extra D segments are removed, thereby, deleting elements with appropriately spaced RSS for secondary rearrangements. However, an alternative means of V_H replacement by V to VDJ rearrangement has been reported in transformed cell lines (3,4), transgenic mouse models (5), and also recently in human germinal center B cells (6) and B cells in rheumatoid arthritis synovium (7). This type of rearrangement is mediated by embedded cryptic RSS within the V_H gene, resulting in "pseudo-hybrid" junctions (5) in the center portion of the V_H gene. Although detection of pseudo-hybrid junctions within V_H gene in these experimental and in vivo systems led to the conclusion that H chain receptor editing could occur, there has been no direct evidence that this phenomenon occurs in human developing B cells as an additional mechanism of achieving self-tolerance.

We have found previously in the analysis of human fetal λ repertoire that receptor editing was actively occurring (8). Therefore, to find the evidence for H chain receptor editing in human developing B cells, we analyzed V_HDJ_H rearrangements obtained from human fetal liver and bone marrow B cells. We have found evidence of receptor editing at the H chain loci, resulting in hybrid V_H genes in human fetal B cells and this phenomenon appears to be RAG mediated. We also found evidence of an additional novel mechanism of H chain receptor editing in which the hypervariable region of CDR2 is replaced, likely by a gene conversion mechanism.

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Materials and Methods

Cell preparation and sorting. Single cell preparations were made from fetal liver and fetal bone marrow at 18 wk of gestation. Fetal cells were obtained as described previously (8). All tissue collections and processing were done in accordance with policies established by the institutional review board for human experimentation at the University of Texas Southwestern Medical Center. Mononuclear cells were enriched by ficoll-hypaque density-gradient centrifugation as described (9). The cells were then stained with a phycoerythin-labeled anti-CD19 mAb (Becton Dickinson, Mountain View, CA) and a FITC-labeled anti-human IgM mAb (Caltag, Burlingame, CA). The individual $CD19^+/IgM^+$ and $CD19^+/IgM^-$ B cells were sorted using a FACStar^{Plus} flow cytometer (Becton Dickinson, San Jose, CA) and genomic DNA was obtained as described previously (8).

PCR amplification and sequence analysis. PCR amplification included an initial primer extension preamplification (9) and subsequent nested PCR steps (9). Rearranged V_HDJ_H genes were then amplified as described previously (9). After column purification of PCR products (GenElute agarose spin columns, Supelco, Bellefonte, PA), PCR products were directly sequenced using the ABI Prism Dye Termination Cycle Sequencing kit (Perkin-Elmer, Foster City, CA) and analyzed with an automated Sequencer (ABI Prism 377; Applied Biosystems, Inc., Foster City, CA). For identification of the germline V_H gene segments, the V BASE Sequence Directory (10) was used in conjunction with the software programs Sequencher (Gene Codes Corp., Ann Arbor, MI) and DNASTAR (DNASTAR Inc., Madison, WI). V_H, D, J_H gene nomenclature was adopted according to the V BASE Sequence Directory.

Estimation of Taq-polymerase error rate. The maximal PCR error rate for this analysis has been documented to be 1.0×10^{-4} (11). Thus, few, if any, of the nucleotide changes encountered in this analysis can be ascribed to PCR amplification errors.

Results

V_H gene replacement in fetal B cells is a frequent event. Atypical sequences suggestive of H chain receptor editing were found in the V_HDJ_H rearrangements obtained from fetal B cells. One sequence from a total of 53 $CD19^+IgM^-$ pro/pre B cells from fetal liver and two different sequences from a total of 72 $CD19^+IgM^+$ immature B cells from fetal bone marrow consisted of a 5' region from one V_H gene and the 3' region from a second V_H gene. Another sequence from fetal bone marrow immature B cells contained part of a CDR2 that was derived from an another gene. These examples accounted for 3.2% of the rearranged fetal V_H sequences analyzed. Considering that these atypical sequences are only documented when pseudohybrid joints and partial CDR replacement occur in regions of the gene that are sufficiently different to be detected, finding 4 examples of V_H replacement from a total of 125 fetal sequences suggests that this is a relatively frequent event.

Pseudohybrid junctions found in fetal V_H rearrangements. Three V_H rearrangements with pseudohybrid junctions were found in the fetal B cell repertoire (Fig. 1). In each of these, we were able to identify, cryptic RSS sequences at the junction of the two V_H genes. The cryptic RSS sequences consisted of an heptamer (5'-CACCATA-3'), 13 bp spacer, and a nonamer (5'-CCAAGAACC-3') which was embedded in the conserved FR3 region of the V_H4 gene family between codon 67 and codon 77. One sequence from fetal liver $CD19^+/IgM^-$ pre/pro B cells contained a pseudohybrid joint between V_H4-04 and V_H4-39, and consisted of a V_H4-04 rearrangement in which the portion of the sequence upstream to the cryptic RSS was replaced by V_H4-39 (Fig. 1A). Two sequences from fetal marrow $CD19^+/IgM^+$ immature B cells both contained pseudohybrid junctions between V_H4-b and V_H4-59, and consisted of a V_H4-b rearrangement in which the portion of the sequence upstream of the cryptic RSS was replaced by V_H4-59 (Fig. 1B). Each of these rearrangements was unique as the CDR3s were distinct. When the gene locus relationship was analyzed between the V_H genes forming the pseudohybrid junction, the gene introduced into the initial rearrangement was found to be upstream of the initial V_H gene in each. V_H4-39 is located about 400 kb upstream of V_H4-04, whereas V_H4-59 is about 200 kb upstream of V_H4-b. Notably, all sequences with pseudohybrid joints were composed of two members of the V_H4 family and formed productive rearrangements.

Gene conversion like CDR replacement is found in the fetal V_H rearrangements. We identified a sequence that exactly matched a known V_H gene except in part of the CDR2 region. The sequence was derived from fetal bone marrow $CD19^+IgM^+$ B cells and utilized the V_H3-11 gene of the V_H3 family. The sequence contained codon 54 to 58 of the CDR2 region completely replaced by a portion of the CDR2 (codons 53 to 58) of V_H gene, V_H3-8 (Fig. 2). The net result was a CDR2 that was one codon longer than that found in V_H3-11. The partial replacement of V_H3-11 by part of V_H3-8 implied that a gene conversion-like mechanism occurred in human fetal B cell development.

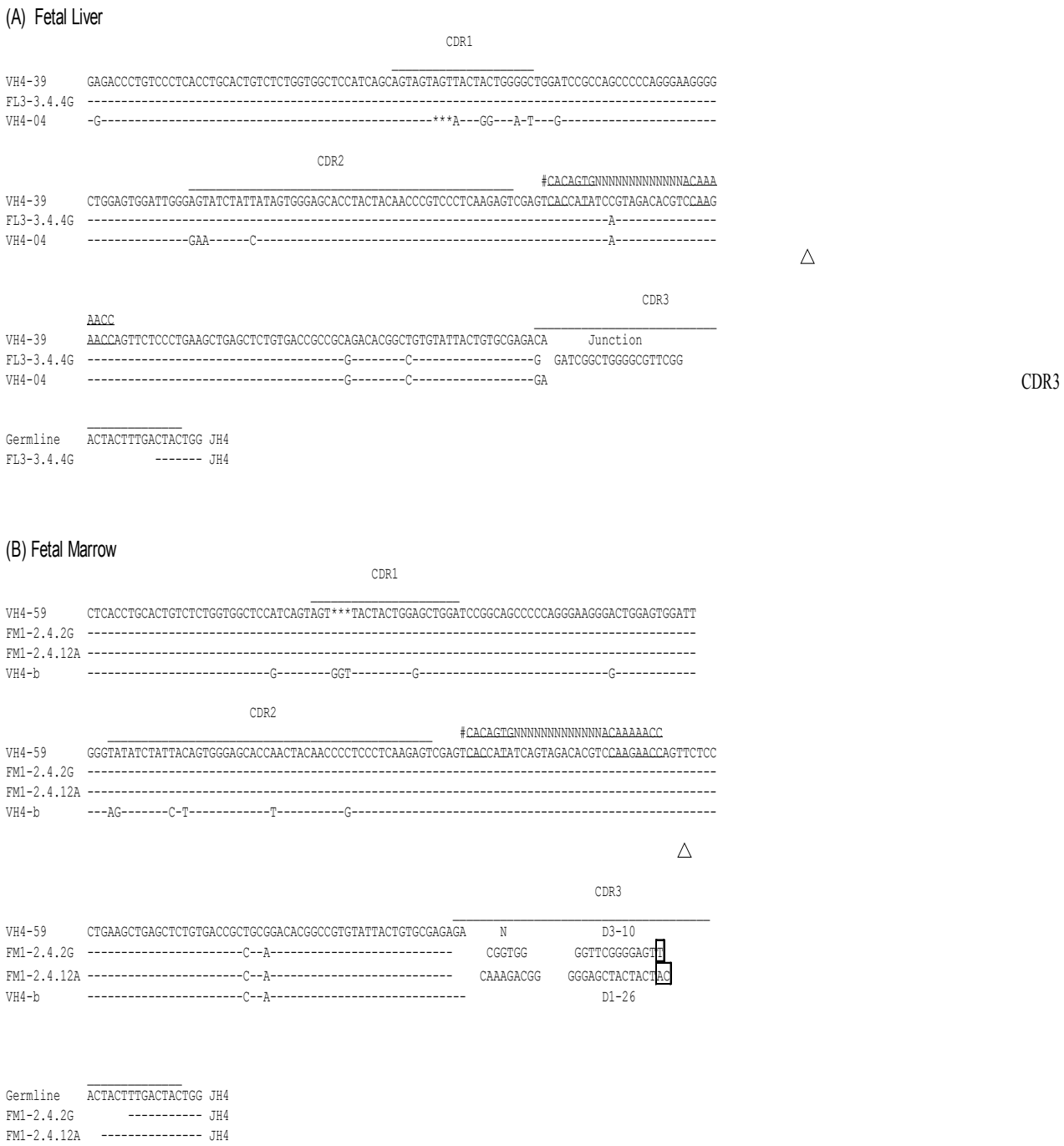

Figure 1. Three examples of pseudohybrid junctions found in fetal V_H rearrangements. Nucleotides homologous to germline V_H genes are indicated in dashes. The white arrowheads mark the proposed junction between the two V_H genes. At the junction of two V_H genes, cryptic RSS (heptamer-13 bp spacer- nonamer) which matches the canonical RSS is underlined. The canonical RSS is shown (#) for comparison. Asterisks mark deletional differences between the two germline genes (V_H4-04/V_H4-39, and V_H4-b/V_H4-59). Nucleotides that cannot be unequivocally assigned to either coding ends (junctional microhomology nucleotides) are boxed at the 5' codong end. A. A V_H hybrid (FL3-3.4.4G) isolated from 18 week fetal liver CD19+/IgM- B cells. The hybrid molecule consists of V_H4-39 upstream of the cryptic RSS and V_H4-04 downstream. B. Two V_H hybrids (FM1-2.4.2G, FM1-2.4.12A) isolated from 18 week fetal marrow CD19+/IgM+ B cells. Both consist of V_H4-59 upstream of the cryptic RSS and V_H4-b downstream.

Figure 2. An heavy chain sequence (FM1-3.3.12A) isolated from 18 week fetal marrow CD19+/IgM+ B cells with a gene conversion event in the CDR2 region. An 18 bp replacement shown in the brackets from the donor gene V_H3-8 (codons 53 to 58) replaces the 15 bp region (codon 54 to 58) of the recipient gene, V_H3-11, resulting in a single codon insertion in CDR2. Nucleotides homologous to germline V_H genes are indicated in dashes. Asterisks indicate nucleotides missing from V_H3-11 that are present in FM1-3.3.12A.

Discussion

Since it is known that the H chain contributes importantly to antigen specificity (12), a mechanism to exchange H chains efficiently would seem to be important. However, H-chain receptor editing cannot be achieved by the normal mechanism of V(D)J recombination. An alternative mechanism, partial V_H replacement, has been suggested by the finding of V_H rearrangements consisting of the 5' portion of one V_H gene and the 3' portion of a second V_H gene, in transformed cell lines (3,4) and transgenic animals expressing autoantibodies (5). Despite these findings, there have been debates about whether H chain receptor editing mediated by RAG proteins occurs in vivo during development of normal B cells, since V_H replacement has not previously been reported in normal developing B cells.

Detailed analysis of V_H rearrangements was performed from the genomic DNA of single IgM^- or IgM^+ B cells from the fetal liver and fetal bone marrow. Since the amplification was carried out from the genomic DNA and from the single cell, the technique allowed us to eliminate the possible bias introduced in analyzing the rearranged Ig repertoire by cloning from multi-cDNA (8,9,11). Amplifying Ig genes from genomic DNA of single B cell has been shown to minimize unusual PCR induced artifacts, such as creation of chimeric molecules or nucleotide exchanges between the mutational variants, known to occur during amplification of c-DNA from bulk populations of B cells (13). Importantly, the sequence error rate using this technique is very low ($<1\times10^{-4}$/base pair), largely because the PCR products are directly sequenced (11). This makes it possible to distinguish minor differences in sequences, permitting a more comprehensive interpretation of the data. In addition, the fetal sequences analyzed contained no mutations, thereby making it possible to interpret sequence differences accurately. This is especially important in identifying V_H hybrid joints, since V_H genes within the same family share more than 80% homology (14), and when there is extensive mutation, it is often difficult to identify the V_H gene segment accurately.

We have used this approach to analyze the human fetal repertoire and have shown previously that receptor editing of λ chain in the fetus is more active than in the adult (8). A number of physiologic events are likely to contribute to the increased λ chain receptor editing in fetus. First, during fetal life, it is likely that the immune system is continuously challenged with autoantigens, creating an environment in which active receptor editing is necessary. Secondly, it is likely that RAG protein expression levels are higher during the immature B cell stage and persist longer in the fetus, creating a favorable situation for receptor replacement to occur.

We found three examples of V_H hybrids, rearrangements in which part of the V_H rearrangement originates from one V_H and rest from another, during the analysis of the human fetal heavy chain repertoire. A number of common characteristics of these V_H hybrids were observed. First, all three V_H hybrids utilized V_H4 gene family member for initial V_HDJ_H rearrangement, and also for V_H replacement. Analysis of the germline sequences of human V_H genes suggested an explanation for the finding that all the V_H hybrids detected involved V_H4 genes. The V_H4 family genes are unique in that they all contain an embedded cryptic RSS in the FR3. Importantly, the embedded RSS in V_H4 family genes are composed not only of an heptamer 5'-(C/T)ACCAT(A/G)-3' but also a 13 bp spacer and a nonamer 5'-C(C/T)AAGAACC. This V_H4 FR3 cryptic RSS meets the minimal sequence requirements for a complete RSS, and therefore is likely to be recognized by RAG proteins. In a canonical RSS, the three nucleotides of the heptamer closest to the recombination site (5'CAC-) are invariant, whereas sequence positions 6 and 7 of the nonamer (5'-AA-) are critical (15). Although other V_H families contain sequences that resemble minimal heptamer sequences, V_H gene families other than V_H4 do not contain embedded sequences that meet these minimal criteria of a complete RSS, containing an heptamer, spacer, and nonamer. Therefore, V_H genes other than V_H4 family members are less likely to participate in this form of V_H receptor editing, either as a donor or as a recipient. A second feature of these V_H hybrids was that the replacement gene was always upstream of the initially rearranged V_H gene. This finding further supports the conclusion that these V_H hybrids resulted from secondary rearrangements, since during the initial rearrangement, V_H genes downstream of the rearrangement are deleted, leaving only V_H genes upstream of the initial rearrangement available for the secondary rearrangement. Finally, V_H hybrids were always found to be productively rearranged. Since V_H hybrid formation does not involve the CDR3 region, and would be anticipated to involve only initially productive rearrangements, the result would be another productive rearrangement if there were no modifications of the pseudohybrid joint. In classic hybrid joints derived from artificial substrates, a considerable degree of end processing was found (16). The signal end is usually kept intact, but the coding end exhibits modifications, with loss or gain of nucleotides. However, in the current study, no end processing was observed. This is probably because nucleotide addition or deletion could cause a frameshift and result in nonproductive heavy chain rearrangements that would be deleted from the repertoire. This implies that V_H replacement may be a more frequent event than observed.

The finding of Wilson et al (6) that the increased frequency of somatic mutation found in the IgD^+germinal center (GC) subpopulation correlated with the frequency of hybrid V_H rearrangements suggested that the activity of the somatic hypermutation machinery rather than RAG enzymes might be a possible mechanism for V_H hybrid formation. Similarly, the V_H1 hybrids found in the rheumatoid arthritis synovium were all found in mutated rearrangements (7). Our data clearly demonstrate V_H hybrid rearrangements in fetal sequences with no mutations, indicating that they were likely to be mediated by RAG proteins, that are highly expressed during fetal development (8). Moreover, the finding that V_H hybrid joint formation was only found to involve V_H4 genes with complete cryptic RSS, including an heptamer, spacer, and nonamer, further supports the conclusion that these are likely to result from RAG-mediated events.

It is notable that one V_H4 hybrid was found in a $CD19^+IgM^-$ fetal liver pro/pre B cell. As this cell was IgM^-, it is unlikely that autoreactivity of the expressed BCR was the trigger for receptor editing. Rather, this suggests the possibility that the persistence of RAG expression during pro/pre B cell development may induce secondary rearrangements independent of BCR signaling. Such secondary rearrangements may be a normal mechanism of generating increased receptor diversity during B cell ontogeny, independent of the requirement to purge the repertoire of autoreactive B cells.

We also found a unique sequence suggesting another mechanism of V_H replacement. The sequence contained partial replacement of the V_H3-11 gene CDR2 region by a gene segment from another V_H gene, V_H3-8. A striking finding was that the region between codon 54 to 58, which was replaced in the V_H3-11 gene, is one of the known major ligand contact sites of V_H genes (17). It is likely that the CDR2 replacement in the V_H3-11 gene would alter the major antigen contact region and change the antigen specificity of the gene. It is noteworthy that this event occurred in a V_H3 family member. Evidence of H chain receptor editing by pseudohybrid joint formation was not found in the sequences utilizing V_H3 family genes. This is likely to be related to the observation that V_H families other than the V_H4 do not contain an intact cryptic RSS composed of a heptamer, spacer, and nonamer. Since the V_H3 family is the most frequently used among the V_H families in the normal adult (9), autoreactive receptors utilizing the V_H3 gene are bound to occur. In these circumstances, autoreactive receptors containing the V_H3 gene would be expected to have a mechanism

for receptor revision, but perhaps involving something other than pseudohybrid joint formation because of the absence of a complete RSS. Our results suggest that replacing the ligand binding region of CDR could be an alternative mechanism for heavy chain receptor editing in situations where complete RSS within the V_H gene are not present.

The mechanism that could account for this type of CDR replacement is unknown. However, the nature of the sequence replacing the initial CDR2 region, which is not from a random gene but from an another V_H gene, gives some clues to the possible mechanism. In chickens and rabbits, gene conversion between genomic V elements occurs routinely and is involved in the generation of Ig V gene diversity (18,19). In addition, gene conversion appears to modify the human pre B receptor (20). In Ig genes, gene conversion is a nonreciprocal recombination event in which upstream V genes donate portions of their sequence to the rearranged V(D)J gene (18). Gene conversion occurs in *cis* and is directional (19), and is homology dependent with genes with greatest homology to the recipient gene being used as a donor gene (21). Although gene conversion may introduce point mutations at the border of the converted segment, gene conversion is a rather precise mechanism involving DNA tracks of various lengths, from 10 to more than 100 bp (22). Gene conversion events can occur without change in alignment but also can cause a shift in alignment resulting in codon addition or deletion (18). Our sequence contained an 18 bp segment from the donor gene, V_H3-8, replacing a 15 bp region of the recipient gene and thereby creating a 1 codon insertion in CDR2. The donor gene V_H3-8 is an unmapped V_H3 family gene that is highly homologous to the recipient gene, V_H3-11. These findings suggest that CDR replacement found in this sequence may be the result of a gene conversion mechanism similar to that found in chickens and rabbits. This is of importance since it may be the first example of authentic gene conversion in human Ig genes. In chickens and rabbits, gene conversion may serve as an additional mechanism to generate diversity, whereas in humans, its role may be primarily to edit autoreactive receptors.

The current data clearly demonstrate evidence of receptor editing at the H chain locus in normal human fetal B cells. Our results showed two possible mechanisms for heavy chain receptor editing in human developing B cells: RAG-dependent hybrid V_H gene formation and CDR replacement by a gene conversion mechanism. Heavy chain receptor editing appears to be a frequent event during fetal B cell development, actively revising the receptor specificity to generate additional diversity as well as to provide self-tolerance and survival of B cells in an autoreactive environment.

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