Secondary mechanisms of diversification in the human antibody repertoire

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INTRODUCTION

A diverse antibody repertoire is a principal component of humoral immunity and is critical to the development of functional adaptive immune responses. Generation of this repertoire diversity is accomplished primarily through two mechanisms: recombinational and somatic hypermutation (SHM). These two mechanisms produce massive diversity within antibody complementarity determining regions (CDRs), which form the primary antigen contact sites. In addition to enhanced diversification resulting from V(D)J recombination and SHM, there are several secondary mechanisms that, while less frequent, make substantial contributions to antibody diversity including V(D)J recombination (or D-D fusion), SHM-associated insertions and deletions, and affinity maturation and antigen contact by non-CDR regions of the antibody. In addition to enhanced diversification, these mechanisms allow the production of antibodies that are critical to response to a variety of viral and bacterial pathogens but that would be difficult to generate using only the primary mechanisms of diversification.

V(D)J RECOMBINATION: FOLLOWING THE 12/23 RULE

Since the discovery that recombination activating gene (RAG)-mediated recombination of variable (V), diversity (D), and joining (J) genes generates virtually unlimited sequence diversity in the antibody repertoire, there have been numerous attempts to develop models that can explain how sequence diversity is generated. The V(D)J recombinase is a eukaryotic enzyme that mediates the recombination of V(D)J gene segments to produce antibody diversity. The V(D)J recombination process involves the cleavage of a double-stranded DNA break at the recognition site. While V(D)J recombination and SHM are the primary mechanisms for diversification of the human antibody repertoire, there are several secondary mechanisms that, while less frequent, make substantial contributions to antibody diversity including V(D)J recombination (or D-D fusion), SHM-associated insertions and deletions, and affinity maturation and antigen contact by non-CDR regions of the antibody. In addition to enhanced diversification, these mechanisms allow the production of antibodies that are critical to response to a variety of viral and bacterial pathogens but that would be difficult to generate using only the primary mechanisms of diversification.

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Kurosawa and Tonegawa, 1982). Recombination thus proceeds in a step-wise fashion, with D-JH recombination preceding V(D)J recombination, resulting in a complete heavy chain variable region (Alt et al., 1987; Schatz et al., 1992). A single recombination event joins the light chain V and J gene, and pairing of recombined heavy chain and recombined light chains results in massive diversity within the unmutated antibody repertoire.

**NON-12/23 RECOMBINATION: V(D)J AND DIRECT V_{H-JH} RECOMBINATION**

Direct V_{H-JH} joining and V(D)J recombination (also referred to as D-D fusion) are in direct violation of the 12/23 rule, but such recombination events have been demonstrated in both in vitro and in vivo systems (Sanz, 1991; Kiyoi et al., 1992; Raaphorst et al., 1997; Koralov et al., 2005, 2006; Watson et al., 2006). Even in model systems designed to induce such recombination events, however, non-12/23 recombinations are much less efficient than recombinations that adhere to the 12/23 rule (Akira et al., 1987; Hesse et al., 1989; Akamatsu et al., 1994).

V(D)J recombinations are the result of an aberrant recombination process by which two or more D genes are joined into a single recombinant. The joining of two D genes, which are flanked on both sides by 12-bp RSSs, can only be accomplished in clear violation of the 12/23 rule, but recombined antibody genes in this configuration have now been isolated by numerous investigators. While V(D)J recombination typically results in an unusually long heavy chain CDR3 (HCDR3) region, the use of two D segments is not the primary mechanism by which long HCDR3 loops are generated (Briney et al., 2012a). Long HCDR3s typically are generated by the use of longer D and J segments and long non-templated junctional regions. The precise order of events during the V(D)J recombination process is unclear: it is not known whether V(D)J recombinants are produced through an additional D-J recombination following the initial D-JH recombination, or whether D-D fusion occurs before, even long before, the D-JH recombination. V(D)J recombinations have been estimated by some to occur in as many as 5–11% of all recombinations (Sanz, 1991; Kiyoi et al., 1992; Raaphorst et al., 1997), but the true frequency of V(D)J recombinations is difficult to determine. Identification of V(D)J recombinants relies on the accurate detection of two diversity genes within a single recombinant, but N-addition mimicry of diversity gene segments, which is genetically indistinguishable from true V(D)J recombination, likely inflates many published estimates of V(D)J recombination (Watson et al., 2006). Recent work, which leveraged high-throughput sequencing and a stringent filtering process, placed a lower bound of the frequency of V(D)J recombinants in the human peripheral blood repertoire at approximately 1 in 800 B cells (Briney et al., 2012b).

The occurrence of direct V_{H-JH} recombination, like V(D)J recombination, requires clear violation of the 12/23 rule, since both V_{H} and J_{H} segments are flanked by 23-bp RSSs. Little is known about the frequency of direct V_{H-JH} recombination in the human repertoire. Several studies of the human CDR3 repertoire that have identified D-D fusions have failed to identify V_{H-JH} recombinants, indicating that if they occur, V_{H-JH} recombinations are likely very rare (Sanz, 1991; Kiyoi et al., 1992; Raaphorst et al., 1997; Watson et al., 2006). This finding is somewhat surprising, since in vitro recombination between two 23-bp RSSs occurred much more frequently than recombination between two 12-bp RSSs (Jones and Gellert, 2002). In contrast to D-D fusions, for which there are several studies on the frequency of V(D)J recombinants in the human peripheral blood repertoire, much of the published work describing in vivo V_{H-JH} recombination relies on transgenic mouse models lacking D gene loci (Koralov et al., 2005, 2006). Since these model systems produce only aberrant recombinants, it is difficult to interpret the resulting data in terms of the likely occurrence and frequency of such recombinants in the naturally occurring circulating B cell repertoire. As with V(D)J recombination, determination of the true frequency of direct V_{H-JH} recombination will likely prove difficult, as extensive check back of D genes during normal V(D)J recombination may appear genetically indistinguishable from true V_{H-JH} recombination and inflate any estimates of the frequency of V_{H-JH} recombination.

**NON-12/23 RECOMBINATION: V_{H REPLACEMENT AND RECEPTOR REVISION**

V_{H} replacement is a process by which a secondary V_{H}-V(D)J recombination can occur, resulting in replacement of the variable gene while preserving the original D-JH recombination. V_{H} replacement, which is thought to be a form of heavy chain receptor editing, differs from light chain receptor editing, although both typically occur early in B cell development (Prak and Weigert, 1995; Nemazee and Weigert, 2000). Light chain receptor editing results in an entirely new V_{L}-J_{L} recombination through the recombination of a V_{L} gene segment upstream of the original recombination with a J_{L} gene segment downstream of the original recombination (Papavasiliou et al., 1997; Ritter and Nemazee, 1998). Thus, light chain receptor editing proceeds without violating the 12/23 rule. In contrast, V_{H} replacement involves V_{H}-V(D)J recombination, which results in retention of the original D-J_{H} junction and replacement only of the V_{H} gene segment (Kleinfield and Weigert, 1989; Nemazee, 2006). V_{H} replacement utilizes a cryptic RSS (cRSS) found near the 3′ end of most human variable genes (Radic and Zouali, 1996), and this cRSS is used to recombine with the normal RSS at the 3′ end of the invading variable gene. The cRSS contains a heptamer sequence, but lacks an identifiable nonamer or spacer sequence, and recombination with the cRSS is inefficient, much like other forms of non-12/23 recombination (Koralov et al., 2006; Lutz et al., 2006).

V_{H} replacement also can be distinguished from receptor revision, which is putatively antigen-driven and has not been shown to use the conserved cRSS elements near the 3′ end of the V gene. Instead, receptor revisions are suggested to occur peripherally in mature B cells using alternate RSS-like elements that sometimes contain only the CAC motif found at the 5′ end of most RSS heptamers or the opposite GTG motif found at the 3′ end; the few examples of this phenomenon typically occurred near the middle of heavy chain framework region (FR) 3 (Ishii et al., 2000; Wilson et al., 2000; Lenze et al., 2003). Use of these alternate RSS-like elements results in formation of a hybrid V gene, retaining a substantial portion of the initially recombined V gene,
as opposed to the nearly complete removal of the initially recom- 

combined V gene observed in V_{H} replacement. Because the observed 

receptor revision events occurred in stretches of sequence simi-

larity between V genes, it has been proposed that these revisions 

may instead be polymerase chain reaction (PCR) artifacts caused 

by incomplete recombinant amplification followed by priming of 

a different V(D)J recombinant with the partially amplified frag-

ment, resulting in a hybrid sequence (Darlow and Stott, 2003). 

In approximately half of all identified receptor revisions in these 

studies, the invading V gene is located downstream of the vari-

able gene used in the initial V(D)J recombination, which would 

not be possible using the proposed receptor revision mecha-

nism. Inter-chromosomal recombination has been proposed as the 

mechanism for these out-of-order receptor revisions (Wilson et al., 

2000). More recent work has shown that receptor reversions are 

not observed when amplifying from single B cells (Goossens et al., 

2003), providing further evidence that the previously observed 

receptor revisions may be an artifact of PCR amplification of 

multiple antibody sequences from bulk B cells. 

It is thought that V_{H} replacement, like other forms of recep-
tor editing, occurs primarily in the immature B cell population to 

rescue non-functional or autoreactive recombinants (Zhang et al., 

2004; Lutz et al., 2006), but some studies suggest that V_{H} replace-

ment may be possible in mature B cells (Nikula et al., 1996; Han 

et al., 1997; Papavasiliou et al., 1997; Hertz et al., 1998; Nussen-

zweig, 1998). Somewhat paradoxically, V_{H} replacement, which is 

pursued to be a primary mechanism for resolving self-reactive 

combinations, can itself result in antibodies with autoreac-
tive characteristics (Klonowski and Monestier, 2000; Zhang et al., 

2003). V_{H} replacement was observed first in transformed murine 

pre-B cells (Kleinfield et al., 1986; Reth et al., 1986), with sub-

sequent studies identifying V_{H} replacement in vivo (Taki et al., 

1993; Chen et al., 1995). In the most informative work done on 

V_{H} replacement in the human repertoire, a genetic finger-

print of V_{H} replacement was identified in the human peripheral 

blood repertoire (Zhang et al., 2003). Identification of V_{H} replace-

ment events in the peripheral repertoire relies on detection of 

short pentameric sequences that are located between the cRSS 

and the 3′ end of V genes. These pentamers remain even after 

V_{H} replacement, providing an identifiable remnant of the replaced 

V gene. Short pentameric sequences are easily mimicked through 

random N-addition, making reliable detection of V_{H} replacement 

difficult. Therefore, estimates of V_{H} recombination frequency in 

the peripheral blood repertoire have varied widely, from 5 to 22% 

of the total repertoire (Zhang et al., 2003; Korolov et al., 2006; 

Watson et al., 2006). 

**SOMATIC HYPERMUTATION** 

In humans and in mice, diversification of the secondary anti-

body repertoire, which arises in response to antigenic stimulus, 

is accomplished primarily through SHM (Brenner and Milstein, 

1966; Kleoo, 1994). Naive, antigen-inexperienced B cells undergo 

the SLM process upon recognition of an infectious agent. It is 

through the SLM process, which occurs primarily in secondary 

lymphoid tissues, that hosts mutate the variable region of their 

antibody genes (MacLennan et al., 1992; Li et al., 2004). Many of 

these mutations have no effect on antigen recognition and many 

have deleterious effects on either antigen recognition or proper 

folding of the antibody protein. Some mutations, however, pro-
duce antibodies with improved affinity for the target pathogenic 

epitope (Casali et al., 2006). Thus, the SLM process provides 
a positive selection of high-affinity antibodies that are 

characteristic of a mature immune response (MacLennan, 1994). 

Many components of the SLM machinery are known, but 
the complete process and the mechanisms by which it is targeted 

specifically to the immunoglobulin loci are still poorly understood. 

SHM introduces point mutations at a frequency of approximately 

10^{-3} mutations per base pair, which is about 10^{6}-fold higher than 

the rate of spontaneous mutation in other genes (Rajewsky et al., 

1987). Mutations begin approximately 150-bp downstream of the 

transcription start site and the mutation frequency decreases expo-

nentially with increasing distance from the transcription start site 

(Rada and Milstein, 2001). Activation-induced cytidine deami-

nase (AID) is required for SLM and initiates the SLM process by 

the deamination of C nucleotides (Muramatsu et al., 1998, 2000). 

Deamination results in a U-G mismatch, and several possible pro-
cesses result in the error-prone repair of the mismatch. Although 

the precise mechanism(s) responsible for error-prone repair dur-
ing SLM are not known, several DNA repair mechanisms have 

been shown to be critical to the SLM process, including base exci-
pion repair and mismatch repair (Phang et al., 1998; Rada et al., 

1998; Wiesendanger et al., 2000; Di Noia and Neuberger, 2002; 

Zheng et al., 2005). 

**SOMATIC HYPERMUTATION-ASSOCIATED INSERTIONS AND DELETIONS** 

Although the SLM process typically results in single nucleotide 

substitutions, deletion of germline nucleic acids or insertion of 

non-germline nucleic acids does occur in association with SLM 

(Goossens et al., 1998; Wilson et al., 1998a; Bemark and Neu-

berger, 2003). These insertions and deletions (indels) are rare, with 

SHM-associated (SHA) indels estimated to be present in 1–6.5% 

of circulating B cells (Goossens et al., 1998, 1999a; Wilson et al., 

1998a; Bemark and Neuberger, 2003). Short SHA indels are much more 

common than long SHA indels, with most insertions and dele-
tions being 1–2 codons in length (Goossens et al., 1998; Wilson 

et al., 1998a; Bemark and Neuberger, 2003). Although infrequent, 

SHA insertion and deletion events add substantially to the diver-

sity of the human antibody repertoire (Wilson et al., 1998b, de 

Wildt et al., 1999; Reason and Zhou, 2006). 

Somatic hypermutation-associated insertions and deletions 

do also have been shown to play a critical role in the antibody 

response against viral and bacterial pathogens, including HIV, 

influenza, and Streptococcus pneumoniae (Zhou et al., 2004; Walker 

et al., 2009, 2011; Wu et al., 2010a; Krause et al., 2011; Psychal 
et al., 2011). Of particular interest, structural analysis of an SHA 

insertion in the anti-influenza antibody 2D1 identified a sub-

stantial structural alteration induced by the insertion (Krause 
et al., 2011). This insertion, although located in a FRs, caused a 

large conformational change in a CDR and allowed antibody– 

antigen interactions that were sterically hindered without the 

insertion-induced conformational change. In addition to 2D1, the 

extremely broad and potentially neutralizing HIV antibody VRC01 

contained a six nucleotide deletion in the CDR1 of the light
chain (CDR-L1; Wu et al., 2010a). This SHA deletion shortened the CDR-L1 loop, thereby removing potential clashes with loop D of the HIV envelope protein and allowing direct interaction between the HIV antigen and the CDR-L2 loop of VRC03 (Zhou et al., 2010).

**ANTIBODY COMPLEMENTARITY DETERMINING REGIONS**

Antibody CDRs (also referred to as hypervariable regions) are the primary region of antigen recognition, contain extensive sequence diversity even among germline genes, and are targeted preferentially for affinity maturation, making them the most variable regions of the antibody gene (Capra and Keoh, 1975; Kabat et al., 1992). There are several structural and genetic reasons for the preferential targeting of CDRs by SHM. Genetically, SHM is known to preferentially target the WRCY hotspot motif (or its reverse complement, RGYW; Dörner et al., 1998), and the frequency of these hotspots is increased in CDRs (Wagner et al., 1995; Shapiro and Wysocki, 2002; Pham et al., 2003). Further, codon usage is biased in CDRs toward codons that are easily mutable, enhancing the likelihood that a nucleotide substitution induced by SHM results in an amino acid change (Motoyama et al., 1991; Wagner et al., 1995; Kepler, 1997). Structurally, the CDRs are largely loop-based, which make them sufficiently flexible to incorporate the substitutions and short indels introduced by SHM without compromising structural integrity. FRs, by contrast, are highly structured and less able to accommodate somatic mutations (Celada and Seiden, 1996).

**AFFINITY MATURATION AND ANTIGEN CONTACT BY ANTIBODY FRAMEWORK REGIONS**

While much affinity maturation is focused on the CDRs, there are other regions that are important to antigen recognition. T cell receptors (TCRs) contain a fourth hypervariable region (HV4, sometimes referred to as CDR4), which is highly variable, surface-exposed, and involved in superantigen and accessory molecule recognition (Choi et al., 1990; Garcia et al., 1996; Li et al., 1998). We have recently used high-throughput sequencing approaches to determine the sequence of thousands of antibody genes containing SHM-associated insertions and deletions (SHA indels), which revealed significant differences between the location of SHA indels and somatic mutations (Briney et al., 2012c). Further, we identified a cluster of insertions and deletions in the antibody FR3 region that corresponds to the HV4 in TCRs.

Emerging evidence suggests that an HV4-like region may exist in antibodies as well as TCRs. Recent crystallographic work on the anti-influenza antibody CR6261 has shown that the HV4-like region of FR3 was somatically mutated (Throsby et al., 2008) and directly contributed to antigen binding (Ekert et al., 2009). The anti-influenza antibody 2D1 contains a three-codon insertion in a HV4-like region of FR3 which, while not directly involved in antigen recognition, causes a critical conformational shift in nearby CDRs that is required for antigen recognition (Krause et al., 2011). A unique example of HV4-like contribution to antigen recognition is the anti-HIV antibody 21c (Duskin et al., 2010). 21c binds to the HIV co-receptor binding pocket, which is only exposed following binding of CD4, the primary host receptor. Interestingly, while the majority of the binding surface of 21c is in contact with the HIV envelope protein, the HV4-like region of 21c binds to CD4, forming a cross-protein epitope. In addition to 21c, the broadly neutralizing anti-HIV antibody VRC03 contains a surprisingly long seven-codon insertion in the HV4-like region of FR3 (Wu et al., 2010a). Finally, the HV4-like FR3 region of antibody heavy chains of the V13 family has been shown to interact with Staphylococcal protein A, a known superantigen (Dorsett et al., 1996), mimicking the superantigen-binding activity of the HV4 region in TCRs. While the HV4-like regions that have been identified to date are not somatically mutated to the same extent as antibody CDRs, the ability of this HV4-like region to tolerate a substantial number of somatic mutations and genetic insertions suggests the existence of a somewhat flexible region that has an under-appreciated ability to accommodate affinity maturation modifications.

**CONCLUSION**

V(D)J recombination, SHA indels, and antigen contact by non-CDR antibody regions, while secondary to V(D)J recombination and SHM as mechanisms of antibody diversification, contribute substantially to antibody diversity. Each of these secondary affinity maturation mechanisms allows for the generation of unique genetic or structural elements that have been shown to be important to the humoral response against a variety of viral and bacterial pathogens including HIV, influenza virus, staphylococci and pneumococci. These secondary affinity maturation events are much less common than SHM and, as a consequence, are more difficult to study effectively. The advent of next-generation sequencing technology has made it possible to obtain thousands or millions, and soon to be billions, of antibody sequences (Boyd et al., 2009, 2010; Wu et al., 2010b; Prabakaran et al., 2011; Briney et al., 2012d). It is likely that over the coming years, this digital flood of antibody sequence data will allow a much more complete understanding of these secondary affinity maturation events. For example, current technologies for isolating antigen-specific antibodies from human blood or bone marrow cells are relatively inefficient and result in stochastic discovery of unique antibodies. High-throughput sequence analysis techniques now allow comprehensive definition of all expressed antibody sequences in samples, even to the scale of analyzing all antibody sequences in leukopacks containing most of the circulating B cells in an individual at a time point. Novel methods under current development for determining phylogenetic relationships among expressed antibody sequences may allow us to define the path of somatic mutation from unmutated ancestor sequences to the final affinity-matured antigen-specific sequence. Likely, these studies will reveal that B cell clones that develop following antigen stimulation do not follow linear paths of development, but rather diverge into complex families with multiply branched phylogenies. Such studies should greatly broaden our understanding of the molecular and genetic events occurring in the B cell repertoire following antigen stimulation.

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