Evolution and the molecular basis of somatic hypermutation of antigen receptor genes

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Somatic hypermutation of immunoglobulin genes occurs in many vertebrates including sharks, frogs, camels, humans and mice. Similarities among species reveal a common mechanism and these include the AGC/T sequence hot spot, preponderance of base substitutions, a bias towards transitions and strand bias. There are some differences among species, however, that may unveil layers of the mechanism. These include a G:C bias in frog and shark IgM but not in nurse shark antigen receptor (NAR), a high frequency of doublets in NAR hypermutation, and the co-occurrence of somatic hypermutation with gene conversion in some species. Here we argue that some of the similarities and differences among species are best explained by error-prone DNA synthesis by the translesion synthesis DNA polymerase zeta (Pol ζ) and, as suggested by others, induction of DNA synthesis by DNA breaks in antigen receptor variable genes. Finally, targeting of the variable genes is probably obtained via transcription-related elements, and it is the targeting phase of somatic hypermutation that is the most likely to reveal molecules unique to adaptive immunity.

Keywords: evolution of somatic hypermutation; gene conversion; DNA polymerase zeta (Pol ζ); nurse shark antigen receptor; pattern of mutation; translesion synthesis

1. INTRODUCTION

Selection on the vertebrate immune system to successfully counteract unpredictable pathogens has resulted in the emergence of a variety of mechanisms that somatically modify the B- and T-cell receptor variable (V) genes. These somatic mechanisms include V(D)J recombination and, in B cells only, class switch, somatic gene conversion and/or somatic hypermutation of immunoglobulin (Ig) genes. Of these mechanisms, V(D)J recombination is best understood, as the enzymes required to catalyse the reaction (RAG proteins) have been discovered, and many of the DNA repair proteins recruited to complete the reaction have been identified (Schatz *et al.* 1989; Oettinger *et al.* 1990; Alt *et al.* 1992; Gellert 1996). The mechanisms of class switch, somatic hypermutation and somatic gene conversion remain largely unknown. In this review, we focus on phylogenetic aspects of somatic hypermutation of Ig genes and discuss whether its evolutionary tracks reveal some clues of its molecular nature.

2. SOMATIC HYPERMUTATION IN VERTEBRATE PHYLOGENY

Somatic hypermutation has been described in sharks, frogs, sheep, camels, mice and humans and, within the context of gene conversion, in rabbits and chickens (Weigert *et al.* 1970; MacLennan & Grey 1986; Reynaud *et al.* 1989; Short *et al.* 1991; Wilson *et al.* 1992; Hinds-Frey

et al. 1993; Greenberg *et al.* 1995; Reynaud *et al.* 1995; Nguyen *et al.* 2000). With the notable exception of sheep (Reynaud *et al.* 1995), somatic hypermutation in the absence of gene conversion is used to fine-tune the specificity of the B-cell receptor to a particular antigen rather than to generate the primary repertoire (discussed in Diaz *et al.* 1998). While some differences exist among different species, analysis of the pattern of mutations generated by hypermutation of antigen receptor genes suggests that many of the components are probably shared among all vertebrates where it occurs. Across-species similarities suggesting a common mechanism include a preponderance of base substitutions, a bias to generate transitions over transversions, the AGC/T sequence hot spot and some degree of strand bias (Golding *et al.* 1987; Betz *et al.* 1993a,b; Dorner *et al.* 1997; reviewed in Diaz & Flajnik 1998). A common mechanism underscores the importance any differences among species may have in revealing layers of the machinery, as separate evolutionary paths may have optimized different components of the mechanism. Therefore, we will first focus on these differences.

3. ECTOTHERMS DO NOT FORM GERMINAL CENTRES

In endotherms, somatic hypermutation occurs in germinal centres (Apel & Berek 1990). These are transient structures formed upon activation by antigen in peripheral secondary lymphoid tissues such as spleen and lymph nodes and wherein antigen presentation by a network of

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Figure 1. Area of potential secondary structure in the nurse shark NAR variable locus, where three out of the five deletion and/or duplication events occurred and where frequent doublets are found.

follicular dendritic cells drives the selection of high affinity variants (Lindhout & De Groot 1995). It has been proposed that germinal centres provide a structured and compartmentalized environment that optimizes selection for high-affinity B-cell receptors that are not self-reactive (Wysocki *et al.* 1998; Pulendran *et al.* 1997). However, germinal centres have not been found in ectotherms (all vertebrates except birds and mammals) (Clem & Leslie 1971), and data from studies of the nurse shark antigen receptor (NAR) locus suggest that at least some degree of antigen-driven selection occurs in the absence of germinal centres (Diaz *et al.* 1998). Furthermore, lymphotoxin- α -deficient mice do not form germinal centres yet acquire mutations associated with high affinity to the hapten (4-hydroxy-3-nitrophenyl) acetyl coupled to ovalbumin after repeated immunizations (Matsumoto *et al.* 1996). Thus, while germinal centres may not be absolutely required for somatic hypermutation or for the selection of high-affinity variants, they probably facilitate and accelerate affinity maturation, and may also play an important role in creating an environment for a second window of tolerance susceptibility (Linton *et al.* 1991). Absence of germinal centres in ectotherms may be accompanied by a slow maturation process, generation of self-reactive B cells or, alternatively, may altogether be compensated by unique structures.

4. DIFFERENCES IN THE PATTERN OF MUTATIONS ACROSS SPECIES

(a) *Shark NAR: high frequency of doublets*

While the pattern and frequency of hypermutation in the NAR locus is strikingly similar to that of mice and human Ig, adjacent mutations (typically in doublets) are found much more frequently in hypermutated NAR genes (Diaz *et al.* 1999). The occurrence of adjacent mutations, as well as a low level of insertions and deletions, appears to be associated with sequences predicted to form secondary structure in NAR V genes (Diaz *et al.* 1999) (figure 1). The importance of potential secondary structure and hypermutability have been noted previously

(Wilson *et al.* 1998; Storb *et al.* 1998), and it suggests that such sites induce 'stuttering' or stalling of a DNA polymerase or of the transcription machinery inducing error-prone synthesis (Wilson *et al.* 1998; Storb *et al.* 1998; Diaz *et al.* 1999). However, it is also possible that these sequences are frequent targets of nicks or double-strand breaks followed by error-prone synthesis. The high frequency of doublets in shark NAR V genes may be the result of selection to enhance local hypermutability and not an intrinsic difference in the mutation machinery. In support of this notion, we seldom see doublets in the first framework region of NAR V that is devoid of sequences that can generate secondary structure (Diaz *et al.* 1999). This finding is consistent with the importance of this region for tertiary structure of the receptor and inconsistent with adjacent mutations being an intrinsic property of the NAR hypermutation machinery.

(b) *Shark and frog IgM: bias to hypermutate G:C bases*

In mouse and human Ig as well as in the nurse shark NAR, G, A and C nucleotides are mutated at equal frequencies while thymidines are mutated less frequently (Betz *et al.* 1993a,b; Pascual *et al.* 1994; Diaz *et al.* 1999; but see Insel & Varade 1994; Dorner *et al.* 1997). In contrast, the horned shark (*Heterodontus francisci*) and frog IgM display a strong G:C bias (Wilson *et al.* 1992; Hinds-Frey *et al.* 1993). G:C bias in these species parallels that found in some human cell lines (Sale & Neuberger 1998; Denepoux *et al.* 2000) and in mice deficient in mismatch repair (MMR) (Jacobs *et al.* 1998; Phung *et al.* 1998; Rada *et al.* 1998; Kim *et al.* 1999; Wiesendanger *et al.* 2000). The G:C bias seen in shark and frog IgM as well as in human cell lines has been attributed to poor selection, suggesting that the machinery preferentially mutates G:C bases but that this effect is obscured by selection for high-affinity variants in animals that form germinal centres (Du Pasquier *et al.* 1998). However, G:C bias is not a characteristic of the nurse shark NAR (or in the N53 shark L chain; Lee *et al.* 2000) in spite of the absence of germinal centres (Diaz *et al.* 1999). In addition, the pattern of mutation in passenger Ig transgenes of mice and/or humans, the heavy chain intron, non-Ig sequences placed within an Ig construct, and non-productively rearranged Ig genes, do not display a G:C bias (Azuma *et al.* 1993; Betz *et al.* 1993a; Gonzalez-Fernandez *et al.* 1994; Yelamos *et al.* 1995; Dorner *et al.* 1997). Interpreting the G:C bias generated in mice deficient in MMR has been confounded by pleiotropic effects, including microsatellite instability, that may impair proliferation in the germinal centre reaction (Vora *et al.* 1999). Still, it remains unclear why a defect in the ability of B cells to undergo many rounds of proliferation would result in an altered pattern of mutations rather than an overall decrease in mutation frequency. Lymphotoxin- α -deficient mice, which also display multiple defects in the immune response and do not form germinal centres in any secondary lymphoid organ, undergo somatic hypermutation at a lower frequency but without a G:C bias (Matsumoto *et al.* 1996). Thus G:C bias does not always correlate with absence of germinal centre and the G:C bias seen in MMR-deficient mice, together with the shark and frog IgM data, suggests either a direct role of the

MMR machinery in somatic hypermutation or that the MMR deficiency may reveal a transitional stage of the mechanism.

(c) **Chickens and rabbit: somatic hypermutation in the context of gene conversion?**

In addition to V(D)J rearrangement, some vertebrates diversify their repertoire further by somatic gene conversion. Clear evidence for gene conversion has been found in rabbits, chickens, cows and pigs (Reynaud *et al.* 1989; Knight & Crane 1994; Parnig *et al.* 1995; Butler *et al.* 1996). The use of a few V genes as acceptor sequences and a relatively high degree of homology among donor V sequences is common among these species, and may be a requisite for gene conversion as it is a recombination-based process. Interestingly, point mutations are found in the D regions and introns of converted rabbit heavy chain genes with a pattern of mutation similar to that generated by mutation in species with no conversion of their Ig genes (Knight & Barrington 1998). Point mutations are also found in gene-converted Ig sequences from the chicken and again, the pattern of mutation and hot spots are similar to somatic hypermutation in mice, humans and shark NAR (Rogozin *et al.* 1996).

A critical question this data raises is whether the point mutations seen in species that undergo gene conversion represent the concurrent existence of two distinct mechanisms in these species, or whether they reveal common features between somatic hypermutation and somatic gene conversion of Ig genes, an idea previously suggested by us and others (Maizels 1989; Weill & Reynaud 1996; Diaz & Flajnik 1998; Knight & Barrington 1998; Blanden & Steele 1998). Several pieces of evidence point to a direct connection between the two mechanisms:

- (i) transcription and/or transcription-related sequences and V(D)J rearrangement are required for both mechanisms (Thompson & Neiman 1987; Peters & Storb 1996);
- (ii) the timing and sequential accumulation of point mutations in rabbits coincides with those of gene conversion (Lanning & Knight 1997);
- (iii) the phylogenetic distribution of species using gene conversion versus somatic hypermutation is best explained by a mechanism that shares some components (Diaz & Flajnik 1998). If these mechanisms were entirely distinct, the phylogenetic distribution would imply multiple gains or losses of the gene conversion or somatic hypermutation machinery in evolution (figure 2).

5. PHASES OF SOMATIC HYPERMUTATION: WHAT DO THE PHYLOGENETIC DATA TELL US ABOUT THE MECHANISM?

We and others favour a model of somatic hypermutation that is divided into two main phases: (i) targeting of a lesion to the V regions of Ig genes, and (ii) error-prone synthesis and repair of the lesion. Similar to V(D)J recombination, one may expect that the molecules that are unique to this mechanism (and perhaps to Ig gene conversion) are expressed during the phase involving targeting of the lesion. The second phase dealing with the

Figure 2. The sporadic phylogenetic distribution of species that undergo gene conversion versus somatic hypermutation suggest a common mechanism. True phylogenetic distances are not represented by the diagram.

aftermath of the lesion is most likely to be carried out by molecules recruited from the cellular DNA repair machinery. We will deal with the second phase of this theoretical context, as it may help reveal the nature of the lesion and of the phase that is unique to the immune response.

(a) **Lesion aftermath: evidence for the error-prone DNA polymerase zeta**

We and others have previously shown that the pattern of mutations shared by all species where somatic hypermutation occurs is similar to the pattern of mutations of non-Ig pseudogenes acquired during neutral evolution, particularly a bias towards transitions (55–60% of point mutations) (Betz *et al.* 1993a,b; Diaz *et al.* 1999). Spontaneous somatic mutations, such as seen in human diploid fibroblasts, also display a transition bias of similar magnitude (McGregor *et al.* 1991). The transition bias of spontaneous mutations in evolution, somatic tissues, and in somatic hypermutation of Ig genes, is a feature of error-prone DNA polymerases (Kunkel 1985), strongly implicating an error-prone polymerase in somatic hypermutation. The fact that transitions, but not transversions in non-degenerate codons, tend to generate synonymous changes implies selection on the evolution of the genetic code to minimize replacements by error-prone DNA synthesis.

As described, the frequency of doublets in the NAR locus also implicates an error-prone polymerase. Whether adjacent mutations are induced by DNA secondary structure or some other mechanism, doublets in the framework region often occurred in the absence of any other nearby or neighbouring mutations, suggesting that they occurred simultaneously (Diaz *et al.* 1999). This implies extension from a mismatched base pair during synthesis, a difficult task for the high fidelity replicative polymerases, but efficiently done by the error-prone polymerases (Nelson *et al.* 1996). In fact, we propose that mutations in the Ig locus

are probably introduced by any of a number of error-prone DNA polymerases, but stabilized by extension from the mismatched base pair by DNA Pol ζ .

We have previously proposed that the translesion synthesis DNA polymerase zeta (Pol ζ) is involved in somatic hypermutation of immunoglobulin genes (Diaz & Flajnik 1998; Diaz *et al.* 1999). This molecule is an attractive candidate because it is responsible for the majority of spontaneous base substitutions in yeast, which also display a transition bias (Quah *et al.* 1980), and it is highly mutagenic in human fibroblasts following ultraviolet light damage (Gibbs *et al.* 1998). Also, Holbeck & Strathern (1997) have shown that point mutations near double-strand breaks in yeast are entirely dependent on the activity of Pol ζ . The fact that Pol ζ is responsible for the majority of base substitutions, but not frameshifts near a double-strand break, demonstrates that it can induce point mutations in undamaged DNA. Furthermore, these data might account for mutations that occur during gene conversion of Ig genes in rabbits and chickens, as gene conversion is probably mediated by a double-strand break. Therefore, the similarities of the pattern of point mutations in gene conversion and somatic hypermutation strongly suggests a role for Pol ζ in inducing mutations in both mechanisms. Recent data suggest that DNA breaks are induced during somatic hypermutation, although it is not clear whether the breaks are single-strand nicks or double-strand breaks (Sale & Neuberger 1998).

Pol ζ is expressed at very low levels in all tissues, including germinal centre B cells (M. Diaz and M. F. Flajnik, unpublished data). Recent evidence suggests that it is regulated post-transcriptionally (Gibbs *et al.* 1988) but that it may be induced (or activated) under stress responses in mice (Kajiwara *et al.* 1996), an interesting notion since the expression of stress-related genes in germinal centres suggests that this reaction is a stress response (Diaz & Klinman 2000). To test the potential role of Pol ζ in somatic hypermutation, we have developed transgenic mice that express high levels of antisense transcript to the RNA encoding the catalytic subunit. Preliminary evidence suggests that these mice have a 40–50% reduction in the overall frequency of somatic hypermutation and a greater reduction in affinity-enhancing mutations during an immune response (Diaz *et al.* 2000).

The G:C bias seen in some species, MMR mutants, and in human cell lines is also likely to result from the recruitment of DNA replication–repair molecules in response to the lesion. The mutations generated by somatic hypermutation of Ig genes, somehow escape correction by the DNA MMR system and this has prompted some investigators to propose that MMR proteins actually fix the mutations by ‘correcting’ the wrong strand (Casalho *et al.* 1998). If it preferentially fixes mutations at A:T bases, then MMR deficiency would abrogate mutations at these nucleotides, which would explain the absence of mutation at these bases in the MMR-deficient mice. This would imply, however, that mutations at G:C bases escape MMR (or misrepair) by some other mechanism, invoking two stages of the mechanism, as recently proposed (Rada *et al.* 1998). A more trivial explanation to the G:C bias is one where, in the absence of MMR, competing but ineffective DNA

repair mechanisms gain access to the Ig locus and preferentially correct mutations at A:T base pairs (a similar proposal has been made recently by Kim & Storb (1998)). Use of the competing repair mechanism may vary depending on the stage of the cell cycle or whether cells are in stationary phase and this may explain differences among species and cell lines. Alternatively, the G:C bias may originate from an initial priming event at a lesion by one DNA polymerase, followed by extension from the mismatch by a second error-prone polymerase with a different error specificity and involvement with the MMR proteins. However, an extensive search of the literature revealed few examples of G:C bias, the most striking found in yeast strains defective in the double-strand break repair protein RAD52, where there is an increase in mutations only at G:C base pairs that were entirely dependent on the activity of Pol ζ (Roche *et al.* 1995).

The likely involvement of a translesion synthesis polymerase in somatic hypermutation prompted us to suggest a model where frequent stalling of the replication fork in the Ig locus—by secondary structure, and/or a transcription complex, or as suggested by others, breaks (Maizels 1989; Sale & Neuberger 1998)—recruits the activity of Pol ζ (Diaz & Flajnik 1998; Diaz *et al.* 1999). The requirement of transcription-related sequences (Azuma *et al.* 1993; Betz *et al.* 1994; Giusti & Manser 1994; Bachl & Wabl 1996; Peters & Storb 1996) but dispensability of the variable gene sequence (Yelamos *et al.* 1995) suggests that transcription elements recruit the machinery to the locus, while secondary structure enhances local hypermutability. The evidence connecting gene conversion to somatic hypermutation, as well as direct evidence from hypermutating cell lines with constitutive expression of terminal deoxynucleotidyl transferase (TdT) suggests that breaks are a feature of this mechanism (Sale & Neuberger 1998). Whether the transcription machinery *per se* rather than transcription regulatory sequences is required to target the breaks to Ig variable genes, remains an open question likely to reveal features and/or molecules that are unique to somatic hypermutation of immunoglobulin genes.

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