A Case Of Convergence: Why Did a Simple Alternative to Canonical Antibodies Arise in Sharks and Camels?

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Introduction

The success of our adaptive humoral immune response, mediated by B cells, relies on its capacity to produce multiple antigen-recognition specificities against any possible invader. This antigen-recognition specificity is attributed to antibodies, which display remarkable flexibility and diversity. That diversity is generated by cutting and pasting of immunoglobulin (Ig) gene segments during B-cell development to generate functional variable (V) genes, which are selected from two separate regions of the genome to yield so-called “heavy” (H) and “light” (L) chains (Figure 1; see Section 1 in Text S1). Ig H and L chain V genes have been expanded in various vertebrate taxa to yield multiple families and subfamilies [1]. Given the obvious advantage of the diversity afforded by such multichain antibodies, it was extremely surprising to discover that some species—notably cartilaginous fish (holocephalins, e.g., chimeras, and elasmobranchs, e.g., sharks, skates, and rays) and camels (e.g., camels and llamas)—developed functional H-chain-only antibodies (HCAbs) (Figures 1 and 2) [2,3]. Loss of the combinatorial H-L diversification of heterotetrameric (H2L2) antibodies should result in a handicapped, less diversified antigen-binding repertoire in HCAbs; yet, contrary to expectations, high-affinity, antigen-reactive HCAbs can be elicited to all studied antigens [4,5]. Even more peculiar, the HCAbs function alongside conventional H2L2 antibodies. What explains this remarkable evolutionary convergence of functional HCAbs in sharks and camels? What forces drove such distantly related species to produce antibodies with this simple structure? And, if they are so effective why is their prevalence not more widespread in evolution?

Occurrence of Functional Heavy Chain-Only Antibodies in Camelids and Shark

The antigen-binding fragment of camelid HCAbs consists of a single domain known as a variable domain of the heavy chain of HCAbs (VHH) (Figure 1) [2]. It was demonstrated that HCAbs from dromedaries (one-humped camels) infected with trypanosomes are capable of associating tightly and specifically with these parasite antigens [2]. Subsequently, HCAbs were elicited to many different foreign antigens. HCAbs are present in all living species of the mammalian family Camelidae, but not in other ungulates (hoofed mammals). Approximately equal levels of HCAbs and H2L2 antibodies are present in camel blood [6]. The origin of camelid Ig genes encoding HCAbs is unambiguous. Phylogenetic analyses have demonstrated that the HCAb-dedicated γ genes were derived from γ genes coding for conventional antibodies. The genes emerged and diverged ~25 million years ago [6,7], after Tylopoda split from other mammals (60–80 million years ago) and before the camel and llama speciation (~11 million years ago) (Figure 2) [6]. Also, the VHH germline genes descended from the classical variable region of a heavy-chain (VH) genes, and the diversity (D) and JH genes are even shared in the formation of the VH or VHH domains. Thus, the emergence of HCAbs was a relatively recent event in these species.

In the nurse shark (Ginglymostoma cirratum), a novel secreted antigen receptor was reported and named the new antigen receptor (NAR) [3]. It too is composed of two covalently associated H-chains with no associated L-chains, but having six domains (Figure 1). Since the molecule shares several functional features with Ig isotypes, it was renamed IgNAR [8], and its N-terminal V domain is known as V-NAR. Later studies showed IgNAR to be induced by immunization with protein antigens with similar kinetics as in a typical IgM response [9]. Although the origin of shark IgNAR is poorly understood, IgNAR is found in all elasmobranchs and thus emerged at least 220 million years ago (Figure 2). However, the single-domain V is also found as a T-cell receptor (TCR) in all living cartilaginous fish (so-called NAR-TCR) [10,11], showing that this immune system feature appeared at least 350 million years ago (Figure 2). In addition, V-NAR gene clusters actually are present in the TCR-Δ loci of all cartilaginous fish and thus it remains uncertain whether they originated as a TCR, an Ig, or perhaps a common antigen receptor ancestor [10].

Convergent Evolutionary Steps to Generate Functional Heavy Chain-Only Antibodies

Besides the phylogenetic origins, the most significant question regarding molecular convergence is: “What are the critical events or properties that must be introduced to produce a functional HCAb?” We think that three (and perhaps four) crucial requirements must be fulfilled to obtain homodimeric H-chain

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Abbreviations: C, constant; D, diversity; FR, framework region; H, heavy; HCAb, heavy-chain antibody; Ig, immunoglobulin; J, joining; L, light; NAR, new antigen receptor; TCR, T-cell receptors; VH, variable region of a heavy chain; VL, variable region of a light-chain; VHH, variable region of a heavy chain of a heavy-chain antibody

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antibodies: (1) the first constant domain (CH1) within the H-chain that normally serves to anchor the L chain should be absent or extensively modified to prevent its retention within the endoplasmic reticulum (see Section 2 in Text S1) [12]; (2) an extensive and diverse primary repertoire of single V-domains must be generated from which to select the most appropriate binders (see Section 3 in Text S1 on how single V domain sequences are diversified) [6], and the selected autonomous V domain should be able to bind antigen with high-affinity-like conventional antibodies; (3) the V domain must be soluble (i.e., nonsticky) in the absence of a variable region of a light-chain (VL) partner (see Section 4 in Text S1) [13]; and (4) although not absolutely required, noncanonical disulfide bonds should be present that stabilize the single V domain in the absence of VL, and to diversify the repertoire of the antigen binding site (see Section 3 in Text S1) [6,14,15].

These requirements underlying the molecular convergence between the HCAbs of camels and the Ig-NARs of cartilaginous fish have been met, resulting in the appearance of functional HCAbs that have converged at multiple levels: (1) The structural level: Camelid HCAbs and Ig-NARs are both homodimeric H-chain antibodies devoid of any L-chain. (2) The sequence level: There is a subtle amino acid sequence convergence of the VH and V-NAR antigen binding domains (i.e., increased frequency of charged and polar residues at the “former VL interface”). (3) The mechanistic level: The strategy whereby H-chain avoids interaction with L-chain (absence of CH1), and the mechanisms that have been employed to create a highly variable antigen-binding repertoire in absence of the H-L combinatorial diversity. (4) The functional level: The VH of camelid HCAbs and the V-NARs of shark IgNARs target similar epitopes that are distinct from the preferred epitopes of classical H\textsubscript{2}L\textsubscript{2} antibodies.

**What Drove the Evolution and Selection of Homodimeric H-chain Antibodies?**

Such a molecular convergent evolution of homodimeric HCAbs in species as diverse as camels and sharks suggests that the immune systems of their ancestors faced comparable stress, and in order to be selected, propagated, and fixed in the population these molecules likely provided a strong advantage in protecting the animal. Although multiple hypotheses can be proposed, the driving force remains elusive. Possibilities could be an L-chain variant prone to malfunction (by amyloidosis for example [16]), or an L-chain used as a coreceptor for a virus that was B-cell tropic. In such an event, the humoral immune system would benefit from the development of antibodies without L chains. Once HCAbs were designed and widespread in the species, the defective L-chain was no longer expressed; or it was modified leading to alleviation of its malfunctioning; or to prevention of virus recognition so that the viral threat disappeared.

In an alternative and possibly more realistic scenario, HCAbs emerged from the need (or advantage) for the adaptive humoral immune system to develop antibodies with a smaller, prolate (rugby ball-like)–shaped, single-domain, antigen-binding V domain that could complement canonical antibodies with their planar or concave binding sites (see Section 5 in Text S1). Such minimal-sized, single-domain antigen-binding fragments have smaller footprints on the antigen and provide access to cryptic epitopes that are inaccessible to VH/VL-based antibodies, or to epitopes with a concave architecture, such as the catalytic clefts of enzymes [17]. Indeed, from several crystal structures of shark V-NAR and camelid VHH bound to antigen it seems clear that the HCAbs of camels and sharks are better equipped to target these epitopes [15,18–20].
The antigen-free V-domain [19]. This conformational loop constraint in the antigen-free antibody will lead to a lower entropic penalty upon antigen binding, where it is expected that the loops are somehow fixed in a unique position within the antibody-antigen complex.

Conception of HCAbs in Widely Different Contexts

Camels and sharks, two evolutionarily remote species (Figure 2), display extreme molecular convergence of their Igs. Both species have a dichotomous adaptive humoral immune system with both conventional and homodimeric antibodies and share the features described above. However, the cameld homodimeric antibodies were derived from conventional genes of the IgH locus by a relatively recent adaptation [7], while IgNAR of the shark originated from an ancient, undefined evolutionary event. Shark Ig-NARs might display the primordial condition, although it remains enigmatic how the unassociated V regions of a single-chain antibody could evolve into dimeric Vs; perhaps the high salt/urea conditions described above was one driving force in selecting for a dichotomous immune system.

The question must be addressed: if, indeed, the HCAbs offer a complementary binding site to conventional VH/VL antibodies, why haven’t they been found in all vertebrates? Their presence in all cartilaginous fish, either as a TCR or an Ig [10], is a testament to the unique organization of their antigen receptor genes. While all other vertebrates employ the so-called translocon-type organization (VnDnJnC), cartilaginous fish Ig genes are encoded in clusters (VnDJnC) (Figure 4) [10,11]. Such an organization...
affords the sharks freedom to evolve new Igs “without much evolutionary fuss,” such as germline-joined clusters for generation of binders of conserved antigens, exchange of genes between TCR and Ig loci, and the single-domain V, HCAbs described here. Indeed, in the holocephalin *Hydrolagus colliei* (spotted ratfish) there has been CH1 loss in an IgM cluster concomitant with a CH2 exon duplication, which likely resulted in the emergence of another homodimeric IgM (Figure 2) [23]; the protein encoded by this gene has not been examined, but it is likely to function as an L-chainless, H-chain dimer, on the basis of the criteria detailed above. Single-domain Vs have evolved also in monotremes and marsupials via generation of an entirely new locus, which is a chimera between Ig and TCR loci (Figure 2) [24]. By contrast, the mechanism by which both camelid HCAbs and conventional antibodies are generated from the same locus during B-cell maturation remains elusive. A novel addition to the classical IgM-IgG class switch must have evolved to allow production of these camelid HCAbs [25]. Thus, HCAbs have emerged by convergence at least four times in vertebrate evolution, and we believe that they are clearly useful and, anthropomorphically speaking, all species would love to have them; however, the organization of Ig and TCR loci in all vertebrates besides cartilaginous fish precludes, or makes quite difficult, the capacity to generate them. Consistent with the idea that single-domain Vs are a useful innovation, once they emerged in the ancestral camelid and cartilaginous fish species, HCAbs have been perpetuated as a major immune system attribute in all of the descendant species.

**Conclusions**

We have described the convergent features of single-chain antibodies that permitted their emergence and reemergence and the constraints that may have precluded their existence in all vertebrates. On the basis of recent discoveries, it is possible that they will be found in other species as well, either as an Ig or a TCR. Single-domain Vs have a growing repertoire of uses in both clinical and basic research, a testament to their complement of the conventional VH/VL antibodies; basic research on the animals that have naturally evolved such antibodies will provide refined theories of their dedicated functions, and give greater insight to their emergence several times in vertebrate history.
Gene (or VH4) has to recombine with one D and one J minigene and should be transcribed with the C before transcription occurs with the C. Production of HCAbs, one of the VHH3 genes (or the VH4) has to be rearranged with one of the D and one of the J genes of the same translocon with the C. After rearrangement of these minigenes, cotranscription occurs with the W constant gene or C. Rearrangement of these minigenes, cotranscription occurs with the W constant gene or with the C-NAR genes to produce classical H_L2 antibodies or HCAbs, respectively. There are multiple clusters in the genome of shark and the V minigenes of some clusters belong to either type I, type II (both clustered with 3-D segments), or type III (clustered with 2-D segments) minigenes.

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**Supporting Information**

**Text S1** Section 1: The rationale for the H_L2 antibody-based adaptive humoral immune system in jawed vertebrates. Section 2: Absence of CH1 domain in H chains for HCAbs. Section 3: Generation of a large V domain repertoire in absence of the VH-

**References**

1. Kirkham PM, Mortari F, Newton JA, Schroder HW Jr (1992) EMBO J 11: 603–609.
2. Hamers-Casterman G, Atarhouch T, Muyldermans S, Robinson G, Hamers C, et al. (1993) Naturally occurring antibodies devoid of light chains. Nature 363: 446–448.
3. Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, et al. (1995) A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature 374: 168–173.
4. Muyldermans S, Cambillau C, Wyns L (2001) Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. Trends Biochem Sci 26: 230–235.
5. Dooley H, Flajnik MF (2006) Antibody repertoire development in cartilaginous fish. Dev Comp Immunol 30: 43–56.
6. Conrath KE, Wernery U, Muyldermans S, Nguyen VK (2003) Emergence and evolution of functional heavy-chain antibodies in Cameliidae. Dev Comp Immunol 27: 87–103.
7. Nguyen VK, Su C, Muyldermans S, van der Loo W (2002) Heavy-chain antibodies in Camelidae: a case of evolutionary innovation. Immunogenetics 54: 39–47.
8. Roux KH, Greenberg AS, Greene L, Sreets L, Avila D, et al. (1998) Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular recognition of NAR and unusual mammalian immunoglobulins. Proc Natl Acad Sci U S A 95: 11084–11089.
9. Dooley H, Flajnik MF (2005) Shark immunity bites back: affinity maturation and memory response in the nurse shark, Gnathostoma crarium. Eur J Immunol 35: 936–945.
10. Crocchiolo MF, Salis M, Flajnik MF (2006) An evolutionarily mobile antigen receptor variable region gene: doubly rearranging NAR-TcR genes in sharks. Proc Natl Acad Sci U S A 103: 5036–5041.
11. Flajnik MF, Kasahara M (2010) Origin and evolution of the adaptive immune system genetic events and selective pressures. Nat Rev Genet 11: 47–59.
12. Feige MJ, Groscurth S, Marcinowski M, Shimizu Y, Kessler H, et al. (2009) An unfolded CH1 domain controls the assembly and secretion of IgG antibodies. Mol Cell 34: 569–579.
13. Davies J, Riewland L (1994) ‘Camelising’ human antibody fragments: NMR studies on VH domains. FEBS Lett 339: 285–290.
14. Nguyen VK, Hamers R, Wyns L, Muyldermans S (2000) Camel heavy-chain antibodies: diverse germline V_HH and specific mechanisms enlarge the antigen-binding repertoire. EMBO J 19: 921–930.
15. Stanfield RL, Dooley H, Flajnik MF, Wilson IA (2004) Crystal structure of a shark single-domain antibody V region in complex with lysozyme. Science 305: 1770–1773.
16. Hurle MR, Helms LR, Li L, Chen W, Wetzel R (1994) A role for destabilizing amino acid replacement in light chain amyloidosis. Proc Natl Acad Sci U S A 91: 5446–5450.
17. Lauerwers Y, Arbab Ghaehroudi M, Desmyter A, Kinne J, Holzer W, et al. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. EMBO J 17: 3512–3520.
18. De Genst E, Silence K, Decanniere K, Conrath K, Loris R, et al. (2006) Molecular basis for the preferential clef recognition by dromedary heavy-chain antibodies. Proc Natl Acad Sci U S A 103: 4586–4591.
19. Desmyter A, Transue TR, Ghaehroudi MA, Thi MH, Poortmans F, et al. (1996) Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. Nat Struct Biol 3: 803–811.
20. Stanfield RL, Dooley H, Verdoni P, Flajnik MF, Wilson IA (2007) Maturation of shark single-domain (IgNAR) antibodies: evidence for induced-fit binding. J Mol Biol 367: 358–372.
21. Muyldermans S, Atarhouch T, Saldanha J, Barbosa JA, Hamers R (1994) Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng 7: 1129–1135.
22. Vu KB, Ghaehroudi MA, Wyns L, Muyldermans S (1997) Comparison of llama VH sequences from conventional and heavy chain antibodies. Mol Immunol 34: 1121–1131.
23. Rast JP, Ansemiya CT, Littman RT, Strong SJ, Littman GW (1998) Distinct patterns of IgH structure and organization in a divergent lineage of chondrichthyan fishes. Immunogenetics 47: 234–245.
24. Perza KE, Baker ML, Schwarz RS, Deakin JE, Lindblad-Toh K, et al. (2007) A unique T cell receptor discovered in marsupials. Proc Natl Acad Sci U S A 104: 9776–9781.
25. Muyldermans S, Baral TN, Retamozzo VC, De Baetselier P, De Genst E, et al. (2009) Camelid immunoglobulins and nanobody technology. Vet Immunol Immunopathol 128: 170–183.