Published in final edited form as:

Curr Opin Immunol. 2016 June ; 40: 7–13. doi:10.1016/j.coi.2016.02.003.

DISTINCT ANTIBODY SPECIES: STRUCTURAL DIFFERENCES CREATING THERAPEUTIC OPPORTUNITIES

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Abstract

Antibodies have been a remarkably successful class of molecules for binding a large number of antigens in therapeutic, diagnostic, and research applications. Typical antibodies derived from mouse or human sources use the surface formed by complementarity determining regions (CDRs) on the variable regions of the heavy chain/light chain heterodimer, which typically forms a relatively flat binding surface. Alternative species, particularly camelids and bovines, provide a unique paradigm for antigen recognition through novel domains which form the antigen binding paratope. For camelids, heavy chain antibodies bind antigen with only a single heavy chain variable region, in the absence of light chains. In bovines, ultralong CDR-H3 regions form an independently folding minidomain, which protrudes from the surface of the antibody and is diverse in both its sequence and disulfide patterns. The atypical paratopes of camelids and bovines potentially provide the ability to interact with different epitopes, particularly recessed or concave surfaces, compared to traditional antibodies.

INTRODUCTION

Several species have been immunized throughout the years to produce antibodies for therapeutic or diagnostic applications, as well as for research purposes. Whereas many laboratories developed antibodies in rabbits, mice, rats or guinea pigs to a large extent because these animals were easy and inexpensive to maintain, the first therapeutic antibodies (against venoms or toxins) were usually extracted from immune sera of horse or sheep since these large domesticated animals provide large volumes of serum [1]. However, the practice

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CONFLICT OF INTEREST STATEMENT

VS has equity interests in Sevion Therapeutics which is developing cow antibodies

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of passive immunization of envenomed patients with horse or sheep derived antibodies, or their Fab fragments, is deemed to become obsolete soon [1]. Very often severely envenomed patients that received this life saving treatment suffer from serum sickness, an immune complex hypersensitivity appearing only a few days after exposure to proteins from antiserum from heterologous species [2]. To avoid such problems, there is a growing interest to develop monoclonal antibodies, selected and engineered to be more human-like. Currently, several options are available to arrive at such human-like affinity reagents, that all have some benefits and pitfalls. Highly potent target binders are retrieved from large human synthetic or naive libraries, or from immunized Xenomice or transgenic chickens producing human antibodies, and even from libraries of non-immunoglobulin protein scaffolds [3–5]. Amazingly powerful selection techniques such as phage display, yeast display and ribosome display have been developed to retrieve the best performing binders in a very short period of time [6].

Remarkably, with all these efficient tools (Xeno-mice, ‘naive’ human Fv or Fab libraries, *in vitro* antibody humanization and powerful selection technologies) at hand to produce human-like monoclonal therapeutic antibodies and affinity reagents, antibodies from camelids and bovines seem to cover a unique niche in this competitive antibody field. Indeed, research from the past two decades revealed striking structural differences in antibodies from camels, llamas and cows. Apparently, these structural eccentricities are at the origin for the interaction of these antibodies with epitopes that are less accessible, cryptic, and/or antigenic for other conventional antibody fragments from human, mouse, rabbit or sheep origin [7,8]. Therefore such antibodies constitute a useful complement to the already large variety of therapeutic, diagnostic and research antibodies.

Bovines, camels and llamas belong to the order of Artiodactyls, comprising both, exotic animals (camel, llama, whale, dolphin, hippopotami, giraffe, antelope, etc) and a number of economically very important species (pig, cow, sheep, goat)[9]. Despite their economic importance, a detailed study of their immune system has been largely neglected for a long time. This changed dramatically in the early 1990s when a serendipitous discovery revealed the natural occurrence of functional homodimeric heavy chain antibodies (HCAs) in camelids [10]. Around the same time, studies on B-cells infected with bovine leukemia virus revealed that cattle can produce exceptionally long CDR-H3s [11]. However, it took several years before the advantage of such antibodies was realized and possible practical applications were developed.

CAMELS AND LLAMAS

Emergence of unique functional heavy chain-only antibodies in camelids

After the speciation of Tylopoda from Suidae and Ruminantia, an unknown evolutionary driving force led to the development of a dichotomic adaptive immune system with functional heterotetrameric (H₂L₂) and HCAs devoid of L chains in Camelidae (the only extant family within Tylopoda)[12]. To produce HCAs, some of the constant γ genes in camelids acquired a G to A nucleotide point mutation at the 5' end of the intron between the CH1 and the hinge exons [13]. This mutation inactivated the splicing recognition sequence so that the CH1 region is removed during mRNA splicing [14]. This mutation is shared by

llama and dromedary immunoglobulin γ genes [13,15], which suggests that it emerged in a common ancestor before these species were geographically separated. These species also have in their genome multiple *IGHVH* minigenes, where the encoded conserved, large and hydrophobic amino acids from framework-2 region that normally interact with the VL domain have been substituted by small and/or hydrophilic amino acids [16–18]. It seems that B cells from camelids where these particular *IGHVH* genes participate in V-D-J recombination will eventually produce those unique heavy chain only IgGs because light chains cannot pair with their variable domains. Thus, the main difference between the conventional H₂L₂ and heavy chain only IgG antibodies is that the 55 kDa antigen binding Fab domains of the former are replaced by a single 15 kDa antigen binding domain. This domain is referred to as VHH or Nanobody because of its dimensions in the single digit nm range (Figure 1).

Apart from the framework-2 amino acid differences between a VH (from a conventional H₂L₂ antibody) and a VHH (from a HCAB), additional differences were also noted in the antigen binding loops. These differences are probably necessary adaptations to generate functional antigen-binding antibodies. First, nucleotide mutations in these dedicated *IGHVH* were acquired in the immediate upstream region of the CDR-H1, the first antigen-binding loop [16]. These mutations create additional somatic hypermutation hotspots in this region, so that these amino acids are easily mutated in the affinity maturation process [7,16]. These amino acid substitutions expand the structural repertoire of the first antigen binding loop and provide enhanced adaptability to generate a surface that is complementary to the antigen-surface. Second, the third antigen-binding loop of VHHs is on average longer than the corresponding loop of a VH of conventional antibodies [19]. This longer loop forms a protruding extension from the remaining antigen-binding site, and will increase the potential antigen-interaction surface [20] (Figure 1). However, a longer loop suggests more flexibility in the antigen-free state, and its fixation upon antigen-recognition will cause an entropic penalty for binding. This entropic penalty is minimized by the presence of an interloop disulfide bond, usually between the CDR-H3 and CDR-H1 of VHHs. This interloop cystine will also have a stabilizing effect on the VHH domain [21].

The binding of a single domain VHH with three antigen-binding loops has normally a smaller footprint on the antigen compared to that of paired VH-VL domains with six antigen-binding loops (three per domain) [22]. This doesn't mean that the interacting surface is smaller, as the VHH tends to bind with protruding loops into concave cavities on the surface of the antigen. Apparently, this preferred epitope topology for a VHH is usually not observed in classical antibodies binding to antigen with paired VH-VL domains [20,23,24] (Figure 1, left panel). The paratope of VH-VL pairs from human or mouse antibodies often forms a flat surface to interact with large epitopes on proteins, or might form a groove or cleft to recognize linear peptides or haptens, respectively [23]. However, VHHs also have the capacity to form flat paratopes, sometimes even involving the lateral side of the domain [25–27].

Applications of Nanobodies in research, as diagnostics and therapeutics

The preference of VHH paratopes to interact with cavities on the antigen enables Nanobodies against enzymes to modulate the catalytic activity of the antigen, as the substrate recognition site of enzymes is located within concave epitopes [27–29]. For these reasons, Nanobodies find applications as enzyme inhibitors, or as research tools to unravel an enzymatic mechanism [27,29–31]. Likewise, the interaction between viral ligands and host receptors is also mediated by concave to convex surfaces. Therefore, Nanobodies often compete with virus-to-host receptor recognition as well [32,33].

Multiple crystal structures of Nanobodies in complex with their cognate antigen are available. It is clear that Nanobodies are a useful tool to assist the crystallization of targets that are reluctant to crystallize [24,34–36].

The small size, monomeric and stable behavior of Nanobodies - even within the reducing environment of the cytoplasm - linked with intelligent engineering, opened new avenues for *in vivo* tracing or elimination of their cognate antigen within living cells and organisms to become practical research tools in developmental biology [26,34,37–42],

The very fast blood clearance of labeled Nanobodies seems to be ideal to develop non-invasive *in vivo* imaging tools to diagnose cancer or cardiovascular diseases [43–46]. Furthermore, Nanobodies when functionalized differently (*e.g.* making bivalent, bispecific or biparatopic nanobody constructs or by adding an albumin-binding nanobody to prolong the circulation time in patients, or to conjugate chelators to attach radio-active nuclides for future targeted radio-nuclide therapy), have been reported to be effective for future therapy [47–50]. In addition, Ablynx generated several Nanobody derived constructs that have reached an advanced clinical phase. These products are strengthening the global therapeutic biologic pipeline. However, since these Nanobody based compounds are derived from llama, immunogenic issues (*e.g.* anti-drug antibodies and hypersensitivity reactions) might arise when administered in humans, despite that Nanobodies can easily be ‘humanized’ [51]. At this stage, the immunogenicity of Nanobody-based products is controversial. A study conducted for a GSK drug comprising a human VH domain describes the presence in human sera of anti VH autoantibodies [52]. Such autoantibodies might interact with humanized Nanobodies as well. Likewise, a clinical trial with a tetravalent Nanobody indicated a hepatotoxicity in patients with such pre-existing antibodies [53]. However, the presence of anti Nanobody antibodies could not be detected in patients that received a non-humanized Nanobody [46], nor in patients participating in the Ablynx’ trials [54].

Therapeutic opportunities with llama Fv

In contrast to the IgG constant sequences, the llama VH and VL sequences share a surprisingly high degree of sequence identity with human VH and VL sequences, so that they are virtually identical in structure and sequence [55]. Therefore, a successful strategy was developed at Argenx whereby llamas are used to immunize with human diseased tissue or pathogens of humans to elicit an immune response. The repertoire of Fab fragments from the immune llama are cloned and the antigen-binding Fabs are selected by phage display. The llama VH and VL genes are then extended and expressed with human constant

immunoglobulin genes to produce chimeric IgGs. In this case the antibodies are not HCABs, but *bona fide* H₂L₂ antibodies with heavy chains paired with light chains. These chimeric antibodies are intended to feed a clinical pipeline of antibody-based drugs to treat patients with cancer and severe autoimmune diseases.

BOVINE (COWS)

Natural occurrence of unusual long CDR-H3 in bovine antibodies

Unlike the progress in understanding and engineering camelid antibodies, only recently has the structural and genetic features of cow antibodies come into significant study [8]. Compared to other species, cows are unusual in having long CDR-H3 sequences, with an ultralong subset that can reach up to 70 amino acids long [56,57]. This latter antibody class can make up 10–15% of the repertoire, and appears to utilize only a single V-D-J rearrangement on the heavy chain, which pairs with a limited number of light chains [8]. Diversity of this CDR-H3 region is extensive, and appears to occur through excessive somatic hypermutation, which in many species, including cows, occurs to diversify the primary repertoire prior to antigen encounter [58]. The single *DH2* gene encodes a repeating motif of Gly-Tyr-Gly, whose codons are biased to mutate into cysteine with a single nucleotide change [8,11]. This mutational process, in addition to altering the amino acid sequence, potentially changes disulfide bonding patterns and could substantially change the topology of the paratope by producing novel loop structures [8,59].

Two crystal structures of bovine Fab fragments have been published, both derived from antibody sequences cloned from BLV infected bovine B-cells [8]. The antigens of these antibodies are not known. The structures are remarkable in that the CDR-H3, which have very little sequence identity, are comprised of a similar β -ribbon stalk that protrudes far from the antibody surface, upon which sits a disulfide bonded “knob” domain (Figure 2). Thus, the 60–70 amino acid cow CDR-H3 loop forms an independently folded ‘mushroom’ shaped minidomain, which is distinct from the immunoglobulin domain in which it is embedded [8]. The stalk domains, as well as the framework regions, are nearly superimposable, whereas the knobs are significantly dissimilar. The knob domains of the two antibodies have different disulfide patterns as well as surface shapes and potential. The overall size of the knob domains are 17–20 Å in diameter, and like camelid VHH regions, provide a unique paratope that can potentially engage concave types of epitopes [8].

Antigen binding by this unique class of antibody has not been studied to great depth compared to antibodies derived from other species. Thus far a single antigen specific antibody with an ultralong CDR-H3 has been studied in detail. This antibody targets the bovine diarrheal virus E2–E3 antigen, and all of its binding activity resides in the knob region of CDR-H3 [8]. Removal of the knob alone could abrogate binding, and residues within knob loops important for binding could be identified through alanine scanning mutagenesis [8]. Given that the ultralong CDR-H3 subclass of antibodies appear to utilize the same VH framework and a single light chain, it follows that the other five CDR regions will also be the same amongst all antibodies in the absence of significant somatic hypermutation [8]. This lack of diversity in the non-CDR-H3 CDRs indicates that they may not be involved in binding antigen. Furthermore, structural studies reveal that residues in the

non-CDR-H3 CDRs provide potentially stabilizing contacts with the stalk region of the ultralong CDR-H3 [8]. Therefore, current evidence suggests that the ultralong CDR-H3 subset of antibodies exclusively utilize their ultralong CDR-H3 to bind antigen, with little or no contribution from the other CDRs [8]. Of course further study on other antigen specific antibodies should confirm this hypothesis, and could also shed light on the role of the β -ribbon stalk, if any, on antigen binding.

Therapeutic opportunities with bovine long CDR-H3 containing fragments

Significant interest in engineering cow antibodies has increased in light of the novel structures of the ultralong CDR-H3s. Several peptides have been engineered into the knob region, including GM-CSF, erythropoietin, GLP-1, and ion channel binding toxins derived from venoms [60–63]. Additionally, efforts to humanize the cow scaffold have successfully yielded stable, high-expressing molecules utilizing a human IgG1 scaffold (Bazirgan, de los Rios, and Smider, unpublished). Thus, the potential for using cow-inspired engineered antibodies for therapeutic use is on the horizon. Furthermore, the unusual structural diversity provided by the stalk and knob domain may allow discovery of paratopes that would be impossible to engineer through human or mouse immunoglobulin domain scaffolds. Indeed, deep sequencing of cow heavy chains reveals an abundance of cysteines in a multitude of different positions in the knob, suggesting that the number of potential disulfide bonded loop scaffolds of this small domain may be enormous [8]. The potential to utilize this diversity to identify antibodies to enzymatic active sites, pores, crevices, or other recessed epitopes is just beginning.

CONCLUSIONS

Nature has provided several different paradigms for structural diversity in the humoral immune system. Unique among these are camelids and bovines, where each species appears to have a class of antibody with a paratope that is smaller and may bind recessed or concave epitopes more optimally than traditional antibodies comprised of paratopes derived from typical VH-VL pairs and shorter CDR loops. It is unclear what the evolutionary driver behind these unique antibody systems was, however the substantial structural differences suggest an importance on binding epitopes where a protruding paratope might be more efficient.

In the case of camelids, the unique structural biology of the Nanobody has been taken advantage of in the pharmaceutical industry, with multiple engineered Nanobodies in discovery and clinical development for a multitude of medical conditions. As the basic biology and structural features of the cow antibody system has been elucidated more recently, discovery and engineering efforts are more nascent. However, engineering bioactive peptides into the knob domain and replacement of the stalk with coiled-coil domains has recently been accomplished, and humanization of the variable regions now opens up the possibilities of discovery and development of bovine antibodies with ultralong CDR-H3s for therapeutic use a distinct possibility.

Acknowledgments

The work was supported by Onderzoeksraad Vrije Universiteit Brussel and NIH R01GM105826.

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HIGHLIGHTS

- A fraction of camelid and bovine antibodies possess a unique long CDR-H3 loop
- About 10 amino acids of the camel CDR-H3 protrudes from the paratope
- The 60–70 amino acid cow CDR-H3 loop forms a ‘mushroom’ shaped paratope
- Cow and camelid paratopes seem suitable to recognize recessed or concave epitopes
- Advanced paratope engineering generates unique leads for research and therapy

Figure 1. Structural organization of conventional antibodies, cow F_{AB}s and camelid-derived Nanobodies. The crystal structure of mouse IgG2a (1IGT) (left); a cow F_{AB} (4K3E) (middle) with long CDR-H3 and a dromedary VHH against chicken lysozyme (1MEL) (right) are shown in ribbon presentation and with surface contours superimposed. The light chain domains are in gold. The heavy chain domains are in dark grey. The CDR H1, H2 and H3 loops are in green, orange and red, respectively.

Figure 2.

Ultralong CDR-H3 region of bovine antibodies. A close-up view of the CDR-H3 of bovine antibody BLV1H12 (PDB: 4K3D) illustrating the disulfide bonded "knob" and β -ribbon "stalk" minidomains which protrude far from the typical antibody surface. The heavy chain is colored teal, with disulfide bonds in orange. The surface is shown in partial transparency to illustrate the structural features shown in the ribbon diagram.