Chapter 2

Introduction to Heavy Chain Antibodies and Derived Nanobodies

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Abstract

The immune response of infected or immunized dromedaries contains a diverse repertoire of conventional and heavy chain-only antibodies, both functional in antigen binding. By definition, a heavy chain antibody is devoid of a light chain and in the case of the heavy chain antibodies in camelids the CH1 domain is also missing. Consequently a camelid heavy chain antibody associates with its cognate antigen via a single domain, the variable heavy chain domain of a heavy chain antibody or VHH. An antigen-specific VHH, also known as Nanobody, with excellent biochemical properties can be obtained in various ways. Their recombinant expression provides access to user-friendly tools for a wide variety of applications.

Key words: Camels, Llamas, Heavy chain antibodies, Single domain antibodies, Nanobodies

1. Introduction

Immunoglobulin (Ig) molecules evolve naturally towards molecular recognition units that associate specifically and with high affinity with their cognate target. The basic structure of an Ig, a polypeptide tetramer of approximately150 kDa, comprises two identical pairs of heavy (50 kDa) and light polypeptide chains (25 kDa), linked by interchain disulfide bonds (1). The light chain consists of one variable domain (VL) at the N-terminal end and a single constant domain (CL) at the C-terminal end, whereas the heavy chain contains four or five domains: one variable domain (VH) at the N-terminal end followed by three or four constant domains (CH1, CH2, CH3, and possibly CH4) (see Fig. 1a) (1).

Immunoglobulins are bifunctional molecules that (1) bind antigens and, in addition, (2) initiate secondary biologic processes that are independent of the antigen specificity. These two independent aspects of immunoglobulin function reside in separate regions

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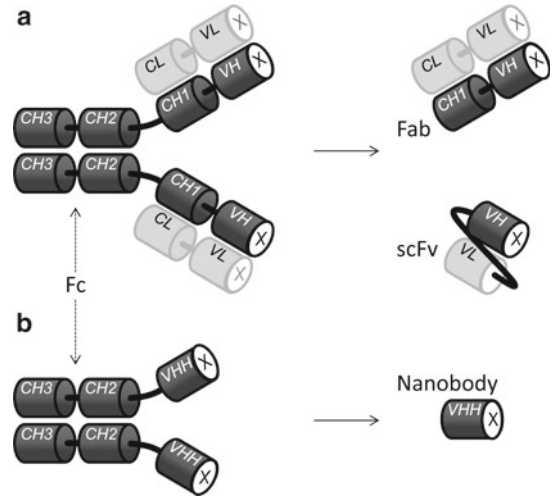

Fig. 1. Schematic representation of (**a**) a conventional and (**b**) a camelid heavy chain IgG antibody and their respective antigen-binding fragments (Fab or scFv for classical antibodies and VHH for HCAbs, respectively). The antigen-binding site of the VH–VL pair or of the VHH (or Nanobody) is denoted by crossed white surfaces.

of each protein. Indeed, these Y- or T-shaped molecules contain two antigen-binding fragments (Fabs), linked via a flexible hinge region located between the CH1 and CH2 domains, to a constant Fc region, responsible for effector functions. The flexibility of the hinge, conferred by a typically loose secondary structure, enables the two Fab arms to move relatively freely with respect to each other. This composition in independent modules and the overall structure of Igs is remarkably well conserved among mammals.

Throughout animal evolution, several classes of Igs have emerged; however, it is the IgG class that is the most abundant immunoglobulin in serum of mammals. It is the product of an affinity-matured immune response and thus, in general, highly specific antibodies with high affinity for their cognate antigen are generated. These antibodies are also very stable and easily purified by a variety of techniques of which affinity-chromatography on Protein A or Protein G is most convenient and well established. For all these reasons IgG is the most important class of antibodies from a biotechnological and medical perspective.

1.1. The Fc Fragment

The Fc portion of antibodies, which comprises the CH2 and CH3 domains of both heavy chains, recruits cytotoxic effector functions through antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) (2). In ADCC, antibodies bind to Fc receptors (FcγRs) located in the membrane of various effector cells, such as natural killer cells, dendritic cells, and macrophages, and trigger phagocytosis or lysis of the targeted cells. In CDC, antibodies kill the targeted cells by triggering the complement

cascade at the cell surface. Each Ig class and subclass has a different Fc fragment with its own specific set of properties. The Fc region also mediates the serum half-life/clearance through binding of antibodies to the neonatal Fc receptor (FcRn) by facilitating their recycling and preventing their catabolism (3). Therefore, the half-life of human IgG1 in blood is generally approximately 3 weeks.

1.2. The Antigen-Binding Site

Located at the N-terminal end of each polypeptide chain, the paired VH and VL domains form the antigen-combining site (Fv) of an antibody. Each domain is made of about 110 amino acids and features the characteristic immunoglobulin fold consisting of a sandwich of two antiparallel β-sheets connected by a conserved intramolecular disulfide bond (1). Within each variable domain, three regions are highly variable in length and/or sequence and are referred to as complementarity determining regions (CDRs) or antigen-binding loops. These regions are separated by relatively invariant stretches, known as framework regions that act as scaffold to support the CDRs. Noncovalent association of the VH and VL domains clusters the hypervariable loops at the N-terminal side of the folded Fv fragment to form an extended interface that interacts with the antigen, the so-called paratope (1). At a structural level, the hypervariable regions fold into a limited number of canonical loop structures, determined by the loop length and the presence of conserved residues at key positions within the hypervariable and framework regions (4). For the CDR3 of VH, the most variable CDR in length and amino acid composition, it is more difficult to predict its structure. Antigen binding is mediated by noncovalent interactions that primarily involve amino acids in the CDRs (especially CDR3) of each chain, but nearby residues in the framework regions may also participate in antigen recognition.

1.3. The Generation of Antigen-Binding Site Diversity

The efficiency of the humoral immune response relies on its ability to generate an almost infinite variety of antibody molecules, each having a unique antigen-binding site. Thus, the immune system must have the genetic capacity to produce a very large number of different variable domain sequences. Immunoglobulin *V* genes in the germline, do not exist as intact, functional genes but as linear arrays of widely separated gene segment clusters: variable (*V*), diversity (*D*), and joining (*J*) gene segments (5). The great diversity of the VL and VH sequences results from a (more or less) random rearrangement of these germline gene segments (combinatorial diversity) whereby one single *VH*, one *D* and one *JH* mini-gene are selected from a set of multiple mini-genes to join and to produce a functional VH polypeptide. Similarly, a *VL-JL* joining leads to the VL domain production. Following successful assembly, producing a single functional heavy or light chain gene, the *V(D)J* rearrangement machinery on the second allele is turned off. This allelic exclusion ensures that a single B cell produces only one type of antibody.

An even greater variety in the region arises because the DNA recombination mechanisms are imprecise and occur in conjunction with deletion of nucleotides at the splice junctions of the recombining *VH-D-JH* or *VL-JL* gene segments. In addition, during assembly of the VH region, nontemplated nucleotides (called N-region) are often added by terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase, at the junction of the *V*, *D*, and *J* segments (6). Moreover, coding joints also contain short deletions and palindromic nucleotide segments. This junctional diversity is achieved at the CDR3 which is a major determinant of the specific interaction between antigen-receptor and antigen (1). Additional diversification of the antigen-binding site (VH–VL) may be introduced by somatic hypermutation (SHM). These mutations are not entirely random as they target preferred sequence motifs known as mutational hotspots (7). These hotspots occur preferentially near or within the antigen-binding site and consequently influence the affinity of the resulting immunoglobulin for its target antigen (8). Individual cells that express higher-affinity mutants have a selective advantage and will proliferate resulting in a continual positive selection for cells bearing higher affinity antibodies, a process called affinity maturation.

2. Heavy Chain Antibodies

The composition and overall structure of antibodies is remarkably well conserved among mammals. Three deviations from the classic heterotetrameric structure have been described over the years, and all of them consist of a homodimer of an immunoglobulin heavy chain. The first described heavy chain antibody is linked to a pathological disorder, known as heavy chain disease (9), and occurs in sera of patients. These truncated antibodies result from a somatic event that removes various parts of the *VH* and *CH1* region from the expressed Ig gene. These human heavy chain antibodies (or those that have been identified in mouse hybridomas) are not functional in antigen binding since the VL and part of the VH domain are missing.

The second type of heavy chain antibodies naturally devoid of light chains were found by serendipity in sera of species from the family of Camelidae (10). The Camelidae (Old World camelids including *Camelus dromedarius* and *Camelus bactrianus*, and New World camelids including *Lama glama*, *Lama pacos*, *Lama guanicoe*, and *Lama vicugna*) belong to the suborder of the Tylopoda that constitutes together with Ruminantia and Suiformes the order of the Artiodactyla. Ruminantia and Suiformes do not possess heavy chain antibodies in their blood, whereas the sera of all camelids contain in addition to the classic isotypes a unique class of

heavy chain antibodies (HCAbs). In sharp contrast with the human HCAbs from patients with heavy chain disease, the camelid HCAbs are bona fide antibodies, which evidently contribute to the immune response of these animals (10). The genetic evidence indicates that the origin of HCAbs is not to be found in remnants of a putative primordial HCAb form but that they are apparently the outcome of more recent adaptive changes occurring in the compartment of the conventional antibodies within the Camelidae lineage (11).

The third type of heavy chain-only antibodies is occurring naturally in shark (12) (see Chapter 3).

2.1. HCAbs in Camelids

Three fractions containing IgG of distinct molecular weight can be isolated from the dromedary serum by differential adsorption on Protein A and Protein G columns. The so-called "IgG1" fraction contains the conventional antibodies comprising two heavy and two light chains. The "IgG2" and "IgG3" fraction contains HCAbs composed of heavy chains that are approximately 10 and 12 kDa smaller than the heavy chain of conventional antibodies (10). This reduced MW of the heavy chain in HCAbs is due to the absence of the entire CH1 domain (see Fig. 1b). The CH1, which functions as an anchor for the light chain in conventional antibodies is encoded in the gene of the heavy chain isotype for the HCAb but is removed during mRNA splicing, due to a point mutation in the splice signal at the 3′ end of the *CH1* exon (13, 14). Hence, the variable domain is joined directly to the hinge region in HCAbs. The removal of the CH1 domain is critical for the secretion of the HCAbs, since intact heavy chains are retained in the endoplasmic reticulum by specific chaperones interacting with the CH1 domain, and it is the displacement of these chaperones by the light chain that allows secretion of classic antibodies (15).

Based on cDNA analysis two isotypes are distinguished within the IgG1 fraction, one encoding a hinge of 19 amino acids (IgG1a) and one encoding a hinge of 12 amino acids (IgG1b) downstream the *CH1* exon. Likewise, the IgG2 fraction from dromedary sera contains two isotypes, one with a hinge of 35 amino acids (IgG2a) and one with a hinge of 15 amino acids (IgG2c). A third type of hinge with 29 amino acids was identified in llama and attributed to the IgG2b subclass (16). Heavy chain antibodies of the IgG3 fraction contain a hinge of 12 amino acids. The relative proportion of heavy chain antibodies to conventional antibodies seems to vary, but an average of 50% of each type is common for Old World camelids, this percentage is somewhat lower (approximately 30%) for New World camelids.

2.2. The Antigen-Binding Site of HCAbs

Since the homodimeric HCAbs lack a light chain and thus a VL domain, the antigen is recognized by one single domain, i.e., the variable domain of the heavy chain of a heavy chain antibody abbreviated as VHH. The recombinant expression of the VHH yields a

soluble single domain antibody (sdAb) fragment with dimensions in the single digit nanometer range, and has been referred to as Nanobody (Nb). These Nanobodies with a MW of only 15 kDa, which is at least half the size of the intact antigen-binding site of a conventional antibody (i.e., the VH–VL pair), are the smallest, intact antigen-binding fragments derived from a functional immunoglobulin (see Fig. 1). Obviously, the VHHs are expected to have acquired important adaptations to remain soluble and functional in the absence of an associated light chain variable domain.

2.3. Sequence Adaptations of VHHs

Nanobodies are distinct from conventional VHs by the substitution of five amino acids that are very well conserved in all VH domains of classic antibodies of vertebrates (17). These VH-VHH hallmark substitutions are as follows: Leu12Ser, Val42Phe/Tyr, Gly49Glu, Leu50Arg/Cys, and Trp52Gly (the last substitution is less well conserved) (numbers refer to the amino acid positions numbered according to IMGT (The International ImMunoGeneTics information system; http://www.imgt.org/)). The substitution of Leu12Ser in dromedary VHH (a considerable fraction of the llama VHH still has a Leu at position 12) is seen as an adaptation to deal with the absence of the CH1 domain (16). The substitution of the hydrophobic side chain of Leu with the smaller, hydrophilic Ser undoubtedly also increases the solubility of the Nanobodies in an aqueous environment. The residues at positions 42, 49, 50, and 52 of the VH domain are part of the large interface with the VL domain. Removal of the VL domain from an Fv would expose a large hydrophobic surface of the VH domain to the solvent, leading to stickiness and aggregation. The hydrophobic to hydrophilic amino acid substitutions observed in Nanobodies explain both their failure to associate with a VL domain and their increased solubility (18).

Three distinctive features of Nanobodies are to be found in the hypervariable regions. First, the CDR1 of Nanobodies is extended towards the N-terminal end (16). The greater variability in this region compared to the corresponding region in VH domains results from the presence of mutational hotspots for SHM imprinted in the germline *VHH* sequences at the codons for residues 28 and 30 (19). This hypervariability suggests that these residues of a VHH participate in antigen binding and that the somatic mutations in this area will be selected during the affinity maturation process. Second, Nanobodies have on average a longer CDR3. The average CDR3 length in dromedary VHHs is 18 amino acids compared to 14 and 11 residues in human and mouse VHs, respectively (17). Third, in addition to the conserved intradomain disulphide bond, the CDR3 of Nanobodies also often harbors a cysteine that forms an additional disulfide bond with a cysteine in the CDR1 or the framework-2 (16, 17). This second cystine constrains the antigen-binding loops and restrains the flexibility of the long CDR3 loop, which might otherwise impede the antigen-binding capacity of the paratope (18).

All these adaptations enlarge the antigen-interaction surface of Nanobodies and offer an additional diversity to their antigen-binding repertoire which probably compensates in part for the absence of the VL domain with its three antigen-binding loops.

2.4. Structural Adaptations of VHHs

Crystal structures confirm that Nanobodies adopt a normal immunoglobulin fold consisting of nine β-strands spread over two β-sheets packed against each other and linked with a conserved disulphide bond. The organization of the Cα-atoms within the β-strands scaffold of the Nanobody superimposes nicely with that in a human or mouse VH (17).

The region interacting with the VL in the conventional VH domains is resurfaced by the VHH specific amino acid substitutions clustered in this region and exhibits therefore a quite different architecture. The nonpolar to polar amino acid substitutions (Gly49Glu and Leu50Arg) increase the hydrophilicity of the surface of the VHH. This effect is even enhanced by the rotation of the hydrophobic side chains of adjacent residues to expose their most hydrophilic parts to the solvent, without deforming the Cα backbone (e.g., Trp118 in cAb-Lys3) (18). Furthermore, the substitutions at positions 42 and 52 cause a net shift of the bulky hydrophobic groups towards the centre of the five-stranded β-sheet. In addition, the CDR3 loop usually folds over these residues and makes them solvent inaccessible.

However, the largest structural differences between the VH and a VHH occur at the level of the antigen-binding loops. Indeed, the CDR1 and CDR2 of Nanobodies adopt a larger number of possible loop structures that deviate fundamentally from the canonical loop structures defined for conventional antibodies (4, 20). This additional structural diversity is attributed to the presence of novel residues at key sites for the loop conformation, the variable length of the CDRs and the formation of an interloop cystine between an additional Cys present in the CDR1 or framework-2 and a Cys located in the CDR3. All these features increase the structural repertoire of Nanobodies by allowing a wide variety of geometrical presentations of the paratope.

Apparently, Nanobodies employ different strategies to interact with their cognate antigen depending on the size and type of the target. As expected, planar paratopes are observed to interact with a proteinaceous antigen (21). Nanobodies are also able to form a cavity with their three CDRs to accommodate binding with a hapten (22). Besides the standard architectures of the paratope such as cavities, grooves or flat surfaces, Nanobodies are also able to form large protruding loops (18). For example, part of the long CDR3 of a lysozyme binder folds over the former VL side while the other part protrudes from the remaining paratope and penetrates into the active site of lysozyme. This large convex paratope provides over 70% of the contacts with lysozyme and the interaction area with lysozyme is as large as the interface between an antigen and a

VH–VL pair. This feature allows Nanobodies to recognize epitopes that are usually not antigenic for classical antibodies, such as the catalytic site of enzymes and canyons in viral and infectious disease biomarkers (18, 23). Hence, it seems that the paratope of HCAbs and conventional antibodies recognize different antigenic sites on their target. It is therefore possible that HCAbs have been selected and maintained in the camelid species for a complementary function in their humoral immune response (11).

Despite the single domain nature of Nanobodies with three antigen binding loops in the paratope of which the CDR3 is the most important, their antigen-binding surface is as large as that of a scFv where the paratope is equally spread over the CDRs of the VH and VL domains (24). As a result the antigen specificity and the affinity or kinetic binding properties of a VHH or a scFv with its cognate antigen are within the same range.

2.5. Diversity of VHH in HCAbs

Identification of germline *VH* (approximately 50) and *VHH* (approximately 40) segments in dromedary demonstrates that VHHs, with their characteristic key residues substitutions, are encoded by a dedicated set of *V* genes, and do not result from an ontogenic process of SHM starting from a *VH* gene (19). Additional support for a dedicated set of *VHH* germline genes is provided by the presence of a codon for a noncanonical cysteine at different possible positions in the *VHH* germline segments, but not in the dromedary *VH* germline segments. Both *VH* and *VHH* gene segments are accommodated in the *V* gene cluster of the heavy chain locus and rearrange with the same *D* and *JH* gene segments to form either a conventional antibody or a HCAb (25). These dromedary *VHHs* can be categorized into seven *VHH* subfamilies, based on the position of the extra cysteine within the CDR1 or the framework-2 region and the length of the CDR2 (either 16 or 17 amino acids) (19). Since extra cysteines are rare in llama VHH sequences, they cannot be used as a subfamily-hallmark and alternative subfamily divisions had to be proposed for llama VHHs (25, 26).

The VH and VHH amino acid sequence share a high degree of identity and are most similar (approximately 80% sequence identity) to the human VH of family 3 (16). Dromedary also possesses a *VH* germline gene sharing a high degree of sequence homology with human *VH* of family 4. Surprisingly, although these genes do not comprise the VHH hallmarks (framework-2 and Cys to form an extra intradomain disulphide bond) their V-D-J recombination products can be part of the heavy chain isotypes of both, classic antibodies and HCAbs. However, current data suggests that this *VH-4* gene is only sporadically expressed in dromedary B cells (27). Thus, the preferential employment of one major subgroup (*VHH-3*) might pose restrictions on the complexity of antigen-binding site of HCAbs. However, the family 3 is the most widespread human VH family in terms of both expression and genome

complexity. Random association of heavy and light variable domains contributes considerably to the expansion of the conventional antibody repertoire. It appears that the HCAbs lacking this VH–VL combinatorial diversity developed multiple diversity mechanisms to acquire a complex repertoire of antigen-binding sites. Indeed, analysis of the *VHH* gene segments evidenced that *VHH* genes exhibits a larger diversity than the *VH* segments. This is attributed to a higher frequency of mutation hotspots and DNA recombination signal sequences in *VHH* germline sequences than in *VH* germline genes (19). The variation in sequence is mainly clustered near or within the paratope at codons whose encoded residues are determinants for the antigen-binding loop structures.

In conclusion, the sequence of Nanobodies is readily diversified by the introduction of an additional disulphide bridge, the high incidence of nucleotide insertions/deletions, gene replacement and extensive somatic point mutations (21, 28).

3. Importance of VHH

Antibodies, either polyclonal or monoclonal, from animal or human origin are essential for a very broad range of applications, as a research tool for target detection or purification, or as a medical tool for diagnosis and therapy. Consequently, the demand for a cheap and renewable source of antibodies is continuously increasing (29). The highly organized and modular structure of antibodies provides a great flexibility for their modification and tailoring to meet special requirements imposed by the envisaged application. Advances in antibody engineering allow fine-tuning of the various features of antibodies, such as valency or avidity, stability, intrinsic affinity, and size.

Whole antibodies, with a molecular weight of about 150 kDa, sometimes lead to practical drawbacks, such as a slow production at high cost, or a weak tissue penetration of the antibody. In addition, for a range of applications, such as radioimmunotherapy or in vivo imaging, the Fc-mediated cellular effects or prolonged half-life in blood are usually serious bottlenecks. Consequently, smaller recombinant antibody fragments become top listed as an emerging new class of drugs. The smallest fragment of a classical antibody that retains the antigen binding specificity of a whole antibody is the scFv in which the VH and VL domains are tethered by a synthetic linker. However, the expression of a single variable domain of an Fv (VH or VL) further reduces the size and might in some instances still produce a fragment retaining residual antigen binding (30). Unfortunately, an isolated VH (or VL) domain exposes a large hydrophobic side to the aqueous environment that renders this type of sdAb rather difficult to handle. Nowadays this shortcoming has

been remediated either by a prior selection of a "soluble and well behaving" (human) VH scaffold (31), or after engineering the VL-binding side of the VH domain according to a man-made design (32) or using the VHH hallmarks in the framework-2 as template. Evidently, an alternative, fast, and convenient source of antigen-specific sdAbs consists in the direct cloning and selection of VHHs from affinity matured HCAbs (from an immunized camelid).

Apart from the size advantages, the recombinant expression of sdAbs turned out to be favorable as well, as validated in various expression systems. Nanobodies are preferentially expressed in *Escherichia coli* where they can be produced economically as soluble and nonaggregating recombinant proteins (33). Higher production levels of Nanobodies are obtained in yeast systems such as *Saccharomyces cerevisiae* and *Pichia pastoris* (34). Finally, functional Nanobodies expressed in tobacco plants have been shown to constitute up to about 1.6% of the total leave soluble fraction, demonstrating the feasibility to use transgenic plants as an economic source of Nanobodies (35).

Nanobodies, because of their single domain nature, offer several advantages for biotechnological applications. Libraries of Nanobodies from immunized camels and llamas can be generated through a straightforward cloning procedure (36). The VHHs within these libraries retain full functional diversity resulting in the isolation of high-affinity antigen-binding Nanobodies after phage display. However, a naïve VHH library or a synthetic library on any autonomous (human) VH might also be used to retrieve specific antigen-binders. Also, the higher stability compared to scFvs, with the feature of reversible denaturation offers an additional asset to these Nanobodies (37, 38). This regained functionality after chemical or thermal denaturation is mainly attributed to an efficient refolding. The adaptations at the former VL interface and the packing of extended CDR3 loops against this interface contributes to the domain stability. These adaptations also confer a high level of solubility to these sdAbs. Furthermore, the single domain nature and the small gene fragment size (350–380 bp) of an sdAb facilitate subsequent molecular manipulation to engineer multivalent formats.

The short serum half-life due to a rapid renal clearance might limit the efficacy of Nanobodies in therapeutic applications. Therefore, Nanobodies have been targeted to normally long-lived serum proteins such as albumin or immunoglobulin using bispecific Nanobodies recognizing these serum proteins in addition to the therapeutic target, resulting in increased half-lives (39). The well-known approach of PEGylation was also successfully used to increase the half-life of a foot-and-mouth disease virus-neutralizing Nanobody (40). Constructing multivalent molecules or engineering the Nanobodies back into full HCAbs by addition of an Fc region is a third possibility to extend serum residence times because of the increased size of the molecule or its interaction with Fc receptors (41).

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