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Single Domain Antibodies: Promising Therapeutic Tools in Infection and Immunity

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Abstract

Antibodies are important tools for experimental research and medical applications. Most antibodies are composed of two heavy and two light chains. Both chains contribute to the antigen-binding site which is usually flat or concave. In addition to these conventional antibodies, llamas and other camelids produce antibodies composed only of heavy chains. Camelids produce functional antibodies devoid of light chains of which the single N-terminal domain is fully capable of antigen binding. These single-domain antibody fragments (VHH) have several advantages for biotechnological applications. VHH is easily produced as recombinant proteins, designated single domain antibodies (sdAbs) or nanobodies. They are well expressed in microorganisms. They have a high stability and solubility, small size, refolding capacity, and good tissue penetration *in vivo*. Here we review the property and results of several recent principle studies that open the acuity of using sdAbs for modulating immune functions and for targeting toxins and microbes.

Keywords: Single domain antibodies, Recombinant antibodies, VHH, Nanobody, Microbial production.

Introduction

Camelids (bactrian camels, dromedaries, and llamas) produce functional antibodies devoid of light chains¹. The IgG1 subclass contains the conventional heterotetrameric antibodies composed of two light and two heavy chains, whereas IgG2a, IgG2b and IgG3 are the homodimeric heavy-chain antibodies, devoid of light chain¹. These heavy-chain antibodies also lack the CH1 domain (Fig.1), which in a conventional degree interacts with the VH domain antibody associates with the light chain and to a lesser degree interacts with the VH domain. However, the paired N-terminal variable domains of heavy (VH)

and light (VL) chains are sufficient for antigen binding². Such antibody fragments can be produced as monovalent antibody fragment (Fab) or as single-chain Fv (scFv) where the VH and VL domains are joined by a polypeptide linker.

Well or chestrated mutation and selection mechanisms ensure the preferential expansion of variants that express antibodies with higher affinity to the immunogen. Repeated immunization, thus, generally yields higher quantities and higher affinities of specific antibodies. Hybridoma and genetic engineering technologies can be used to harness and to reformat individual antibodies obtained from immunized animals and even to

reconstruct recombinant molecules semi-synthetically. Methods to isolate antigen-specific VHHs from immune, nonimmune or semi synthetic libraries using phage, yeast, or ribosome display are now well established.

Properties of sdAb

As compare to conventional antibodies, VHHs have been shown to remain functional at 90°C or after incubation at high temperatures^{3,4}. This high apparent stability is mainly attributed their efficient refolding after chemical or thermal denaturation and to an increased resistance against denaturation⁵. The increased apparent stability is probably due to an increased hydrophilicity of the former VL interface region because a "camelized" VH fragment that contains several of hydrophilic amino acid residues of VHHs was more stable than the original VH fragment. In addition to these specific mutations, the packing of extended CDR3 loops against this former VL interface contributes to domain stability. Furthermore, refolding of VHHs only requires domain refolding, whereas conventional antibodies also require association of VH and VL domains. VHHs can also recognize antigenic sites that are normally not recognized by conventional antibodies such as enzyme active sites⁶. This facilitates their use as enzyme inhibitors or in diagnosis of infections. The ability to recognize these recessed antigenic sites has been attributed to their smaller size and the ability of the extended CDR3 loop to penetrate into such sites⁷. However, hapten and peptide-binding VHHs have been successfully isolated using strong selection systems⁸. Because of their small size of about 15 kDa, VHHs rapidly pass the renal filter, which has a cutoff of about 60 kDa, resulting in their rapid blood clearance. In addition, the small size results in a fast tissue penetration⁹.

sdAb properties versus scFv and Fab

Efficient identification of Ag binders	Nb > scFv = Fab
Good expression yield	Nb > scFv = Fab
Good stability	Nb > Fab > scFv
Good solubility	Nb > Fab > scFv
Antigen specific	Nb = Fab = scFv
High affinity for the Ag	Nb = Fab = scFv
Easy tailoring	Nb > scFv = Fab
sdAb target unique epitopes	Nb = scFv = Fab

Method to isolate antigen-specific VHHs

sdAbs are usually generated by PCR cloning of the V-domain repertoire from blood, lymph node, or spleen cDNA obtained from immunized animals into a phage display vector, such as pHEN2. Antigen-specific sdAbs are commonly selected by panning phage libraries on immobilized antigen, e.g., antigen coated onto the plastic surface of a test tube, biotinylated antigens immobilized on streptavidin beads, or membrane proteins expressed on the surface of cells. Several labs have also constructed semi-synthetic libraries by cassette-mutagenesis of the CDR regions. However, sdAbs derived from such non-immune libraries often show lower affinities for their antigen than sdAbs derived from animals that have received several immunizations¹⁰. The affinity of sdAbs from non-immune libraries can often be improved by mimicking this strategy in vitro, i.e., by site directed mutagenesis of the CDR regions and further rounds of panning on immobilized antigen under conditions of increased stringency (higher temperature, high or low salt concentration, high or low pH, and low antigen concentrations). SdAbs derived from camelid hcAbs are readily expressed in and purified from the *E. coli* periplasm at much higher expression levels than the corresponding domains of conventional antibodies. SdAbs generally display high solubility and stability and can also be readily produced in yeast, plant, and mammalian cells¹¹.

Applications

Tumor targeting

sdAbs search maximal tumor load and fastest blood clearance. A clear signal (by binding with fluorescent protein) is seen 1 hr after injecting the labeled nanobody while with classical antibodies we need to wait at least a few days before the background signal is reduced sufficiently¹².

Targeting leukocyte ecto-enzymes

Leukocytes express numerous ecto-enzymes that have their active sites exposed to the extracellular environment. These enzymes play important roles in cell trafficking, inflammation, and apoptosis¹³ for e.g., NAD-dependent ADP-

ribosyltransferases (ARTs, also named CD296) regulate the function of other cell surface proteins by post-translational modification.

Targeting other cell surface proteins

Leukocytes and other cells express numerous other functionally important membrane proteins that are not enzymes, including receptors, ion channels and transporters. Recently, sdAbs directed against the CD16 Fc-receptor on natural killer (NK) cells were developed from an immunized llama to be used in bi-specific formats for recruiting NK cells to target and destroy tumor cells¹⁴.

Application in cancer therapy

Carcinoembryonic antigen (CEA, also named CD66e) is highly expressed on cancer cells of epithelial origin. A CEA-specific sdAb derived from an immunized dromedary (cAb-CEA5) was fused to a β -lactamase, an enzyme that can convert non-toxic prodrugs into potent cytotoxic agents⁹.

Targeting other soluble proteins

sdAbs have been developed against components of the blood clotting cascade and against aggregation-prone proteins implicated in amyloid diseases. An sdAb derived from an immunized llama specifically recognizes the activated form of von Willebrand factor (vWF), a key component of the blood-clotting cascade that mediates the tethering of platelets to the vascular endothelial wall¹⁵.

Molecular assembly vaccines

The higher immunogenicity of repetitive proteins could potentially be exploited also for increasing vaccine efficiency¹⁶. Lumazine synthase from *Brucella abortus* spontaneously assembles into pentamers and decamers and shows very high immunogenicity even during primary immunization.

sdAbs as tools for molecular mimicry

Antibodies directed against the antigen-binding paratope (idiotype) of another antibody. Anti-idiotypic sdAbs were selected from a semi-synthetic phage display library against the idiotype of a monoclonal antibody specific for a linear

tripeptide epitope in the *Plasmodium falciparum* apical membrane protein antigen-1 (AMA-1), which we can use as vaccine¹⁷.

Targeting protein toxins and poisons

Most potent toxins found in nature are enzymes, including proteins secreted by bacteria and the venomous organs of snakes and scorpions. A sdAb against alpha cobra toxin was derived from a llama and engineered into a pentameric format by genetic fusion to the non-toxic B-subunit of verotoxin, yielding a reagent with higher avidity and neutralization capacity¹⁸.

Targeting small molecule toxins and other haptens

Fungi secrete chemical compounds that can be toxic to animal cells. Recently, a sdAb (NAT-267) was developed from an immunized llama against BSA-conjugated Deoxynivalenol known as vomitoxin (300 Da), a mycotoxin produced by common grain molds¹⁹.

Targeting viruses

Viruses often use "hidden" epitopes on the capsid or envelope, e.g., deep invaginations for docking onto receptors on host cells. Several groups have succeeded in raising sdAbs directed against rotavirus²⁰.

Targeting pathogenic bacteria

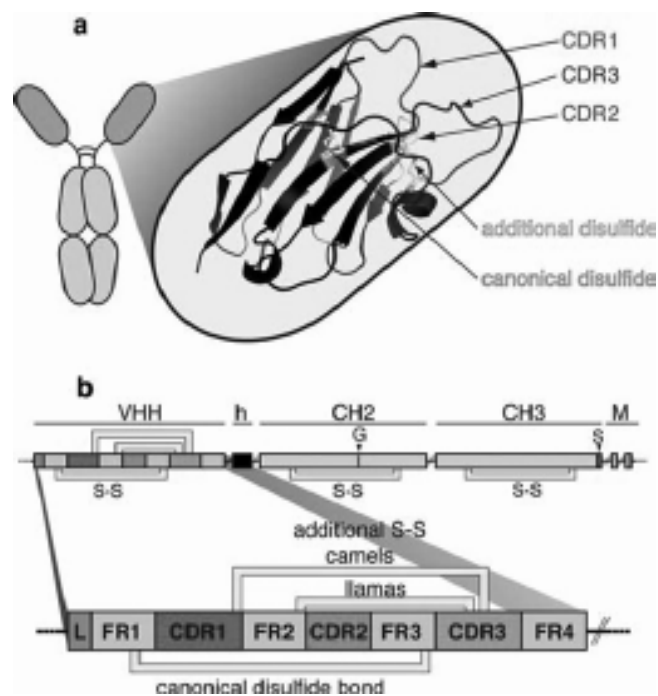
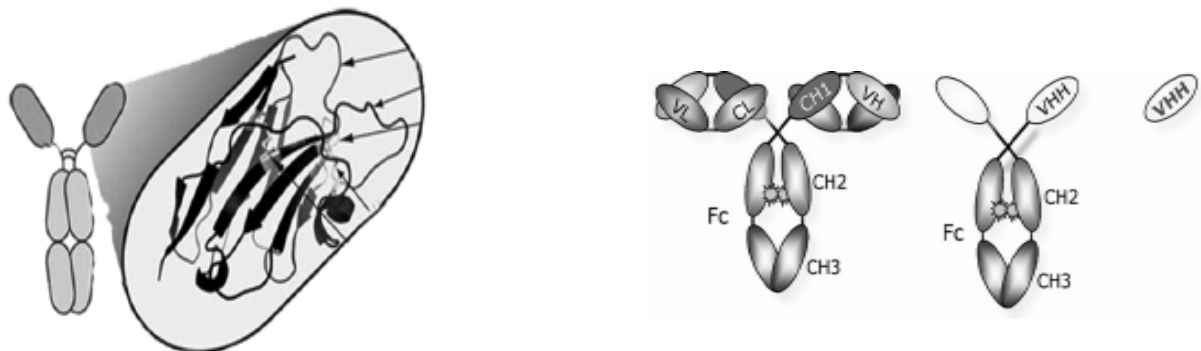
Single domain antibodies have also been raised against bacterial surface proteins with the goals of blocking attachment of bacteria to host cells and/or for more effective delivery of pro-drugs. A VHH (K609) against *E. coli* F4 fimbriae applied at high doses reduced *E. coli* induced diarrhea in piglets²¹.

Targeting parasites

Single domain antibodies are also being developed as antiparasite reagents. The larval form of the pork tapeworm *Taenia solium* is the cause of cysticercosis, the most common parasitic infection of the central nervous system. Immunization of two dromedaries with *T. solium* extracts yielded an sdAb (NbsoI52) recognizing the 14 kD diagnostic glycoprotein Ts14²².

Table 1 Applications of antibodies

Disease	Pathogen	Target antigen	VHH valency	Additional fusion partner	Reference
Sleeping sickness	Trypanosomes	VSG oligomannose	Monovalent	Apolipoprotein L-I	23
Infant diarrhea	Rotavirus	Unknown	Monovalent	None	20
Infant diarrhea	Rotavirus	Unknown	Monovalent	Lactobacillus cell-surface anchor	24
Piglet diarrhea	<i>E. coli</i>	F4 fimbriae	Monovalent	None	21
Caries	<i>S. mutans</i>	I/II adhesion	Monovalent	None	11
FMD	FMD virus	VP1	Monovalent	PEG	8
Sepsis	<i>N. meningitidis</i>	LPS	Monovalent	None	25
Cancer	–	CEA	Monovalent	β -Lactamase	9
Cancer	–	EGF receptor	Bivalent	Anti-albumin VHH	26
Rheumatoid arthritis	–	TNF α	Bivalent	Anti-albumin VHH	27
Brain disorders	–	α (2,3) sialoglyc protein	Monovalent	None	28

**Fig. 1** Structure and composition of antibodies

Conclusions and future perspectives

Since the discovery of heavy-chain antibodies in 1993, the field of single-domain antibody fragments has been rapidly growing. VHHs have many advantages for biotechnological applications. They can be economically produced in micro organisms and have a high stability. Furthermore, they are highly suited for expression as multivalent, including bispecific formats or as enzyme fusions. Conventional whole antibodies occasionally give side effects because of their bivalent nature, which can result in target cross linking, or the presence of the Fc region. Evidently, such side effects are not expected to occur using monovalent VHHs. In vivo studies have underscored the favorable biodistribution of sdAbs, including deep penetration into dense tissues and rapid elimination via the kidney. These features make sdAbs particularly amenable for imaging of tumors and for the delivery of cytotoxic agents. sdAbs should also prove useful for neutralizing soluble extracellular proteins including toxins, cytokines, and blood clotting components.

For intracellular targets, more effective delivery tools will need to be developed for sdAbs to reach the cytosol and other intracellular compartments. Transfection-mediated expression of intracellular sdAbs appears to be a feasible strategy for cell/gene therapy regimens and for transgenic plants and animals. Presently, the immunization of llamas and dromedaries is cumbersome and costly in comparison to immunization of smaller animals. Transgenic mice expressing camelid antibodies, analogous to the transgenic mice expressing human antibodies, could provide more economic sources of sdAbs. Assuming that progress will continue at the present pace, it is likely that the future repertoire of researchers and clinicians will include a battery of sdAb reagents directed against cytokines, ectoenzymes, tumor antigens, toxins, and microbes.

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