

Review

Engineered Bovine Antibodies in the Development of Novel Therapeutics, Immunomodulators and Vaccines

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Abstract: Some bovine antibodies across all classes are unique, such as the CDR3 of the variable heavy-domain (VH CDR3), which is exceptionally long (up to 66 amino acids), unlike most conventional antibodies where the VH CDR3 loops range from 10 to 25 amino acids. The exceptionally long VH CDR3 is encoded by unusually long germline *IGHD* genes together with insertion of novel “a” nucleotide rich conserved short nucleotide sequence (CSNS) specifically at the *IGH V-D* junction. Such an exceptionally long VH CDR3 confers unique “knob and stalk” structural architecture where the knob, formed by intra-VH CDR3 disulfide bridges, is separated by 20 Å solvent exposed stalk composed of anti-parallel beta strands. The substitution of the knob with cytokines, such as, erythropoietin and granulocyte colony stimulating factor 3 (granulocyte colony stimulating factor), results in expression of functional fusion proteins with enhanced pharmacokinetics. The beta stranded stalk can be substituted with other rigid structures, for example, repeat alpha helices to form coiled-coil that mimics the beta-stranded stalk and, thus, opens opportunities for insertion of this structure in the CDRs of antibodies across species. Given the versatility of such a structural platform in bovine antibody VH CDR3, it provides the opportunity for the development of new generation of diagnostics, therapeutics, vaccines and immunomodulating drugs.

Keywords: antibody; scFv; exceptionally long VH CDR3; antigenization; immunomodulation; virus neutralization; vaccine; immunotherapeutics

1. Introduction

Two identical polypeptide chains of an immunoglobulin (IG), heavy (H)- and light (L)-chain, pair together and fold into domains where amino terminal comprises two antigen-binding sites of the same specificity [1]. The constant region domains constitute the carboxyl terminal responsible for a variety of biological effector functions, including the half-life of IG. Unlike other species, for example mice and humans, the bovine antibody repertoire is restrained by limited germline sequence divergence of genetic elements, both at the heavy (IGH)- and light kappa (IGK) or lambda (IGL) loci [2–4]. Based on the number of functional genetic elements, restricted combinatorial diversity ($10IGHV \times 13IGHD \times 2IGHJ \times (8IGKV \times 3IGKJ + 17IGLV \times 2IGLJ = 1.5 \times 10^4)$) is evident in the bovine antibody repertoire, far below the needed ability to recognize possible 10^8 – 10^{11} epitopes. Our laboratory discovered that, in addition to known mechanisms of antibody diversification (e.g., somatic hypermutations, junctional flexibility including N or P additions), generation of an exceptionally long (≥ 50 – 61 amino acids) complementarity-determining region 3 (CDR3) of the variable heavy-domain (VH CDR3) in some cattle antibodies provides a new mechanism of antibody diversification [5–12] that seems to compensate for limited genetic combinatorial diversity for acquisition of required functional antibody-mediated humoral immunity. Such an exceptionally long VH CDR3, with multiple cysteines, expressed in 8%–10% of circulating bovine B cells [6], is the first to be documented for an antibody in a species. The antibodies with such atypical VH CDR3 require restricted and specific V-LAMBDA and VH pairings where IG lambda chain does not make contact with the target epitope but provides the structural support to heavy chain variable domain that principally recognizes the antigen [7].

The exceptionally long VH CDR3 is found in all bovine antibody isotypes [13,14] and appears early during B cell ontogeny [8]. A recombination of germline *IGHV(BF4E9)*, single longest *IGHD2* and *IGHJ(pB7S2)* genes, together with novel conserved short nucleotide sequence (CSNS; 13–18 nucleotides; distinct from N- or P-additions) insertion specifically at IGH V-D junction (non-*IGHV* and non-*IGHD* origin) results in VH CDR3 size beyond germline recombination potential [6,8,10]. The mechanism for generation of such CSNS insertion that enhances VH CDR3 size considerably is yet to be understood. The unique structural features of the bovine antibodies with massive VH CDR3 and corresponding novel configuration of the antigen-combining site offer the potential for developing new generation of antibody-based therapeutics and vaccines [4,15]. Indeed, recent crystallization and X-ray diffraction analysis of bovine antibodies, BLV5B8 and BLV1H12 with exceptionally long VH CDR3 [6] developed in our laboratory, revealed a unique “stalk and knob” structure where the knob is formed by mini-domains created by intra-VH CDR3 disulfide bridges between multiple cysteine amino acids [16]. These VH CDR3 have an unusual architecture since beta strand “stalk” supports a disulfide-bonded “knob” resulting in mini-domains that provide functionally significant configurational diversity. Since antibodies or antibody-fragment related products are among the fastest growing new therapeutics [15], unique genetic, structural and configurational properties of bovine antibodies with

exceptionally long VH CDR3 offer opportunities for the development of new antibody-based diagnostics, immunomodulators, therapeutics and vaccines.

2. Designing Antibody Fragments of Desired Function

The utilization of antibodies in clinical- or immuno-diagnosis, immunomodulation and disease prevention necessitates specific design and construction of antibody fragments or their derivatives to confer a desired function; for example, virus or toxin neutralization (Figure 1a), microbial agglutination and opsonization, complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The development of such tailor-designed antibodies is feasible because specific antibody effector functions are confined to a particular structural domain or specific sequence in the modular structure of an antibody. In this context, antibody fragments (reviewed elsewhere [15,17–20]) comprising VH and VL domains linked together, referred as single chain fragment variable (scFv), are capable of generating a functional antigen-combining site that can recognize an antigen and, also, neutralize a virus or toxin. Given the small molecular size of scFvs, these have a short half-life and are rapidly cleared *in vivo*. This characteristic is advantageous for clinical diagnostic purposes where radioisotope labeled antibody detects antigen via gamma imaging. Alternatively, the serum half-life of the scFv may be enhanced, if desired, through chemical (e.g., PEGylation) or genetic (expression as scFV-FCGRT (FcRn) fusion protein [21]) manipulation. Similarly the opsonization and phagocytic function can be tailored into an scFv by incorporating the relevant Fc domain with the desired target sequence. An insertion of the specific Fc domain at an appropriate location in scFv will also provide ADCC function, e.g., modifications aimed at enhancing FcγRIIIA binding. Similarly, IGHG CH2 or IGHM CH3 domains could be incorporated into scFv to insert complement-binding site with an objective to confer CDC function. The antibody fragments could also be designed to survive in the hostile gut environment, where these could be made resistant to the proteolytic degradation by including the polymeric immunoglobulin receptor (PIGR). From therapeutic perspective, a variety of molecules, e.g., drugs, immuno-pharmaceuticals, toxins, cytokines, *etc.* can be conjugated to the desired Fc subsequent to its expression as fusion protein together with antigen binding function of scFv (Figure 1b) [17,20]. Such variations aimed at conferring a desired effector function would provide useful diagnostics and therapeutics against infectious agents [22–25], cancers [26–28] and immune mediated diseases [29,30].

A unique structural feature of IG V-DOMAIN is that it is composed of hypervariable regions or CDR, three each on VH and VL domains, within relatively conserved four framework regions or FR [1,31,32]. A remarkable characteristic of the FR is that these provide the required molecular microenvironment that permit the CDR to extend into solvent in an immunologically accessible manner while retaining the required configurational structural support. This provides the structural basis of IG variable domain antigenicity that could reside in the FR (public) or CDR (private), termed as idiotype. Indeed, an idiotype (id) acts as IG variable domain epitope present on H and/or L chains forming a regulatory “id-anti-id” network [33–35]. In this context, it is important to note that genetic imprints of all antigens present in the universe (10^8 – 10^{11}) are enshrined within the framework of “internal mirror image” as idiotype in the antibody repertoire. Given this intrinsic characteristic of the IG CDR, these could be genetically antigenized by grafting desired T and B cell epitopes (Figure 1c)

to generate immune response upon immunization [36]. Indeed, viral T and B cell epitopes [37,38], parasitic B cell epitope [33] and CD4 molecule [39] have been used to antigenize IG that induced specific immune response subsequent to immunization. Unlike shorter VH CDR3 of mouse or human IG, exceptionally long VH CDR3 (>50 codons) with multiple cysteine amino acids of bovine IG is better suited for antigenization with configurational B cell epitopes for inducing humoral immunity. The properties of selected configurational epitope grafted onto VH CDR3 of bovine scFv would confer upon it the dominant immunogenicity. Since idiotype of an IG is known to be antigenic [33,34,36–42], grafted epitope(s) onto the CDR will antigenize the IG fragment. Similarly, immunoregulatory molecules such as cytokines could be grafted onto a CDR (Figure 1c) with an objective to enhance their half-life *in vivo* and/or sustained desired regulatory functions. Given the “knob and stalk” structural configuration of the bovine VH CDR3, it would provide the molecular solvent exposed environment sustained by the FR to display desired epitopes or regulatory cytokines in an immunologically-accessible and configurationally-suitable way to achieve the desired function. The antigenized IG or its fragment variable (Fv), by desired epitope grafting, will upon immunization elicit the targeted immune response. Similarly, grafting of an immunomodulating cytokine would engage in effector function with desired enhanced half-life *in vivo*.

Figure 1. Diagrammatic representation of antibody fragment, scFv, design for virus or toxin neutralization function (a); expression as fusion protein with cytokine, e.g., interferon- γ for multiple combined functions, such as, virus neutralization, innate and cell mediated immunity against a virus (b); and epitope or cytokine grafting in the CDR3H of bovine scFv to achieve antigenization or immunomodulation function (c). * Indicates three FR amino acids before VH CDR1.

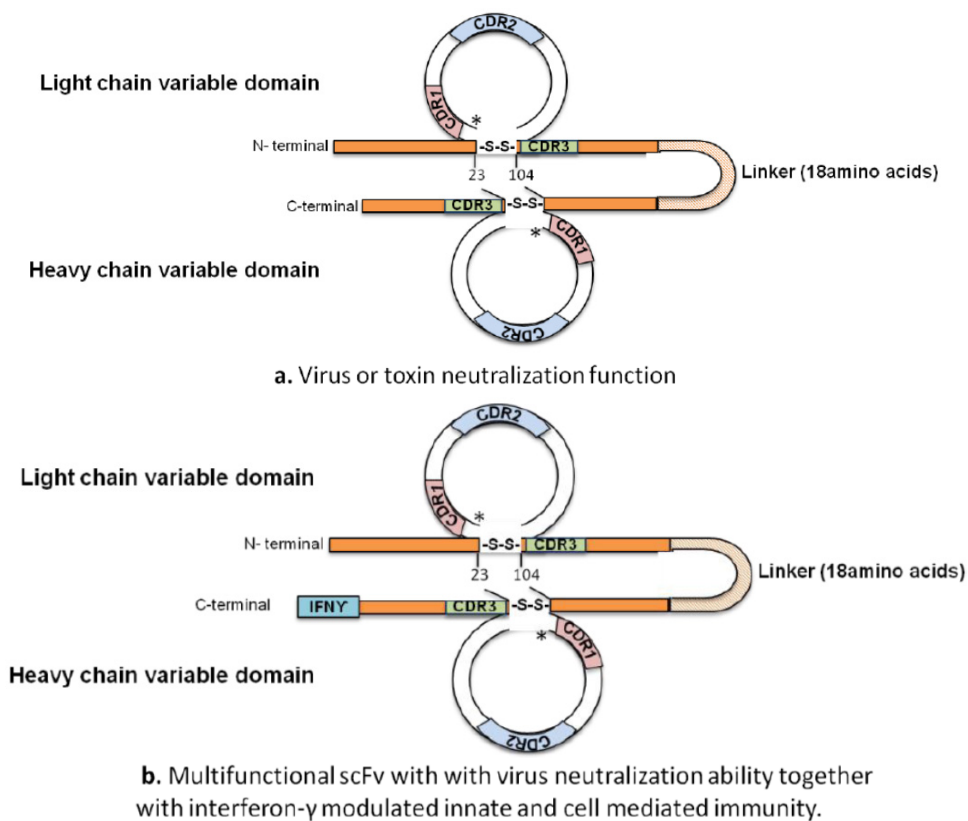
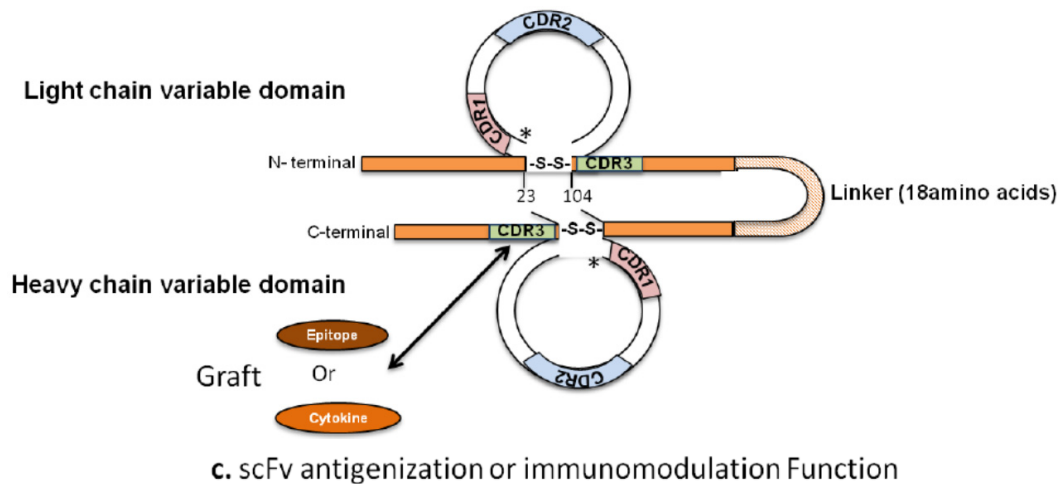


Figure 1. Cont.



3. Development of Bovine Antiviral Antibodies

The development of engineered bovine antibody fragments is yet to be fully realized given its unique structural characteristics, especially the VH CDR3 architecture. A wide variety of scFvs or Fab, originating from hybridomas or phage libraries, for diagnostic and therapeutic purposes, have been constructed against various bacterial and viral pathogens using different expression systems [15]. An scFv with a (Gly₄Ser)₃ linker, against foot and mouth disease virus (FMDV) in cattle, was expressed in *E. coli* as inclusion bodies where the VH and VL were amplified from a murine monoclonal antibody, 1C7, specific for Type O FMDV. As expected, the scFv conserved the same binding specificity as the parent monoclonal antibody [43]. Other similar scFv developed against various bacterial and viral antigens for diagnosis and treatment of human diseases include anti-hepatitis B surface antigen (HBsAg), *Listeria monocytogenes* [44] and *Mycobacterium avium* subsp. *paratuberculosis* [45]. Single chain Fvs have been recently produced against cellular prion protein (PrP^c), one of which inhibits PrP^c translocation to the cell surface [46–48]. Engineered scFv against bovine pathogens hold a strong clinical potential in immunodiagnosics and therapeutics, however, not many of these engineered antibodies have been developed against veterinary pathogens. We previously reported the construction of scFv against bovine herpesvirus type-1 (BoHV-1) [24,25,49]. The anti-BoHV-1 scFv were produced in the *Pichia pastoris* yeast expression system to allow linker-influenced multimerization and glycosylation. Significant *in vitro* virus neutralization was achieved by both the monomeric (18 amino acid linker) and dimerized (7 amino acid linker) scFv [24], though two-fold concentration of monomeric scFv was needed for virus neutralization as compared to dimerized scFv (diabody). A single amino acid change (Asp to Gly) at position 98 according to IMGT unique numbering [31,32] in the VH FR3 of scFv affected the viral neutralization dynamics in a dose-dependent manner [49]. Further, enhancement in the potency of virus neutralization function due to non-covalent multimerization was noted in scFv with two amino acid linker (tri- or tetra-body) [25]. These mono- or multi-meric scFv produced in the *Pichia pastoris* had extensive glycosylation heterogeneity (discussed in [25]) but it did not affect their viral antigen recognition and neutralization function.

Earlier, phage display technology was exploited to generate bovine antibodies against a model antigen glutathione S-transferase [50]. This was followed by the development of a bovine-anti-FMDV

Fab phage library from vaccinated cattle [51]. Single VH-CH1 from Fab were obtained, which showed properties of antigen binding in *in vitro* assays, such as ELISA, western blotting and viral neutralization. It was suggested that the absence of V_L domains in these single chain Fabs did not affect their virus neutralizing function. This finding was attributed to the main role of extended VH CDR3 (24 and 27 amino acids) [51] to virus neutralization. The bovine antibodies, especially those with exceptionally long VH CDR3 [6], provide opportunities for developing novel immunodiagnostics and therapeutics against infectious diseases across various species.

4. Development of Immunomodulating Drugs and Vaccines

Recent X-ray crystal structure analysis of the largest existing antibody (BLV1H12) with an exceptional VH CDR3 size [6,8], revealed a unique “stalk and knob” structure of the antigen-combining site [16]. The knob region was shown to play a crucial role in antigen-binding and, therefore, as suggested previously [6], was exploited for generating fusion proteins with desired pharmacologic properties [52–54]. Since the knob region of BLV1H12 antibody constitutes the antigen-binding site, it was substituted with bovine granulocyte colony-stimulating factor 3 (CSF3; GCSF) and expressed as a stable fusion protein in mammalian expression system. The bovine antibody-bovine GCSF showed potency similar to bovine GCSF, stimulated granulocyte progenitors in dose-dependent manner, significantly increased the serum half life and sustained neutrophil populations for more than three weeks in rodent system [53]. These observations suggested that long-acting therapeutics could be developed using bovine antibody VH CDR3 for fusion of cytokines. Another recent study substituted the knob of BLV1H12 antibody by human erythropoietin protein (hEPO) [52]. The bovine antibody-hEPO fusion protein had potency comparable to the original hEPO, extended plasma half-life and sustained erythropoiesis stimulating activity in mouse model. Thus, generality of knob domain of bovine VH CDR3 for producing biologically active fusion proteins with enhanced pharmacokinetics opens opportunities for developing new generation of immunomodulating drugs. The stalk region of the bovine antibody (BLV1H12) VH CDR3 is a unique and rare protein motif that is seven-residue long solvent exposed beta-strand. Recent studies demonstrate that substitution of beta strand stalk in bovine antibody (BLV1H12) with another rigid structure with a superhelix composed of two or more alpha-helices with a repeated pattern, called coiled-coil, permits generation of functional antibody-bGCSF fusion protein [54]. This opens the opportunity for inserting these unique structures in the CDR of human or other antibodies for fusion of cytokines and growth factors. Such fusion proteins hold promise in developing new therapeutic drugs to combat a variety of infectious diseases, cancers and immune mediated diseases in humans.

Given the size (>50 codons), structural and configurational characteristics of the exceptionally long VH CDR3 in bovine antibodies, these provide suitable platform for antigenization to develop new generation of vaccines. Overall, these observations suggest that the unique structural properties of the VH CDR3 of bovine antibody can be exploited for development of novel next generation of diagnostics, therapeutics, immunomodulators and vaccines.

5. Conclusions and Future Directions

Unlike conventional antibodies where the VH CDR3 loops across species usually range from 10 to 25 amino acids, the bovine VH CDR3 can be massive in size given its extensive heterogeneity extending from 3 to 66 amino acids. The exceptionally long VH CDR3 is encoded by unusually long germline D genes (49 codons) together with insertion of CSNS specifically at the IGH V-D junction. The mechanism of insertion of “a” nucleotide rich CSNS of non-IGHV and non-IGHD origin is yet to be understood. Interestingly, such an exceptionally long VH CDR3 confers unique “knob and stalk” architecture where the knob is separated by 20 Å solvent exposed rigid stalk composed of anti-parallel beta-strands. The substitution of knob with cytokines, such as, erythropoietin and granulocyte colony stimulating factor, resulted in functional fusion proteins with enhanced pharmacokinetics. Further, the ability to replace the beta-stranded stalk with other rigid structures, for example, repeat alpha-helices to form coiled-coil, without loss of function that mimicked the beta-stranded stalk opens opportunities for insertion of this structure in the CDR of antibodies of other species. Given the versatility of such a structural architecture in bovine antibody VH CDR3, it sets new future directions for the development of novel diagnostics, therapeutics, vaccines and immunomodulating drugs.

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Author Contributions

Ashish Sachan and Surinder Saini prepared the background information while Madhuri Koti reviewed the development of engineered anti-microbial antibodies. The conceptual aspects of designing antibody fragments for desired function was provided by Azad Kaushik.

Conflicts of Interest

The authors declare no conflict of interest.

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