Review of: "Bivalent single domain antibody constructs for effective neutralization of Venezuelan equine encephalitis"

Theo Verrips[1]

1 QVQ Holding B.V.

Potential competing interests: The author(s) declared that no potential competing interests exist.

Alphaviruses are single positive-sense RNA viruses surrounded by a membrane. They have a rather complex life cycle involving mosquitos as vector and many vertebrates as hosts, including horses and humans (1). On basis of their clinical syndromes they have been categorized (2). Encephalitic alphaviruses include Venezuelan [VEEV], Eastern [EEEV] and Western [WEEV] alphaviruses. These viruses enter the host cells through receptor mediated endocystosis, although recently slightly different ways of entering their host cells are described (3). The viral membrane bound proteins E1 and E2 play crucial roles in the initial step of entering of the virus into their cognate host cells. Although this genus of viruses is known for quite some time and can be lethal to humans, an approved vaccine to protect humans is not available. Alphaviruses can be spread via aerosols, so sprays containing antibodies may reduce the transmission of the virus to humans and between animals. It is well known that variable domain antibodies, derived from heavy chain antibodies devoid of light chain of Camelidae [VHH or Nanobodies] have the desired properties to be integrated in sprays (4,5). Therefore the group of Ellen Goldman at US Naval Research laboratory, Washington, developed these VHH against Chikununya (6) and VEEV (7). For the latter study they used 3 Llamas to raise these VHH. Their publication contains some intriguing and some neglected aspects. One of the intriguing aspects is that they used 2 [out of 3] llamas that have been immunized nearly a decade earlier with related antigens [Table 1]. Such a long period of rest between two sets of immunization is rather unique and could have provided clues on immune memory processes in llamas. However the plasmas of these animals before the new round of immunization were not analyzed, neither analyses of memory B- and T- cells involved in viral resistance have been performed. But comparison of the immune responses after new rounds of immunization showed that the 2 llama immunized earlier showed clearly better immune responses than llama not immunized with related antigens [although one lama has been used for another immunization [Table 1]. Subsequently they tested the neutralization of the plasma after these new round of immunization on various antigens, including also the more distant alphavirus Chikununya virus [CHIKV] and West Nile Virus [WNV] that belong to the Family of *Flaviviridae*. That the serum from Llama Whisper showed a strong positive effect on WNV was not a big surprise as Whisper was immunized earlier with WNV. The positive serum response of Llama Centavo on CHIKV as such was not a surprise, but the strength of the response indicates that these viruses may contain similar epitopes on their surfaces proteins, in spite of recognizing different host membrane proteins [for VEEV LDLRAD3 and MXRAS for CHIKV]. The fact that Llama Cowboy hardly showed an immune response is surprising as the combination of viral antigens and immunization scheme as used in Liu's study, provoked good responses in all immunizations of llamas with a wide range of viruses, we have performed during 20 years, of which some were published (8-10).

After common phage selection processes on VEEV-TC-83 coated on plates of the 3 separate libraries, the library derived from Centavo was chosen for subsequent studies. They selected VHH of two different V-gene families, notably the KEREF and KQREL families but not of the KEREG and KGLEW families (11,12), which is a small surprise as two - in time separated sets of immunizations with different antigens - normally deliver thousands of VHH derived from all 4 families and also often also different in J-genes are found (13). A closer evaluation of the selected VHH showed that they are quite extensive maturated in the frame works {FW] of these VHH compared to the recent literature data (14) and own data. It is also known that maturation of amino acids of the FW results in an increased number of amino acids from the FW that interact directly or contribute to the binding of the cognate antigen. It was even suggested by Bond et al (15) that in fact FW 3 was an additional CDR. However in the VHH selected in this study, FW2 was the most heavily maturated FW, but it is unknown whether the maturated amino acids are involved in binding to the virus. They also selected a VHH from the Centavo library that was highly homologous to a VHH selected before [CC3]. The newly selected VHH V11A1 and V2C3 differ only at 4 positions from CC3, notably T28A, T58N, V78I and Y82bN and carry all the rare maturations in common with CC3 [A40V; K43S; L47R; A50L]. This indicates strongly a common origin, maybe even a clone already formed during the immunization 8 years ago.

One of the frequently made claims in respect to VHH is their high physical and/or thermostability (16). Stability is very important for the integration of VHH in sprays. This claim is often made, but not supported by a lot of data, so the data provided in this paper are a welcome addition to the total date set on stability of VHH. VHH V2G1 shows a T_m of 73 ^oC and a correct refolding yield of 99%. Whereas the refolding is very good the T_m is just average (16). VHH V8C3 and V3G9 have a rather low T_m of 67 ^oC and low refolding yields of 13 and 17 % respectively. These values indicate low physical stability and most likely bad production in eukaryotes. According to Renisio (17), stability of VHH depends on well fitted hydrophobic core, covered with β-strands on the surface and a restricted number of electrostatic interactions. Indeed V2G1 complies quite well with the rules of Renisio, whereas the VHH with the low refolding yield lack the optimal hydrophobic core and have 3 β strands with sub-optimal sequences.

Of all properties of antibodies the K_D values belong to the most important properties. The paper does not provide data on binding constants, but on neutralization values. Although the latter is {for the purpose the authors had in mind] even more important than K_D values, it is a pity that not always both K_D and neutralization values are given. Large differences in neutralization of VEEV between the various VHH were found. This may indicate that some VHH do not bind very well to the VEEV strain TC-83 or bind to epitopes that are not important for binding of the virus to their cognate receptor protein. They also constructed a number of homo- and [assumed] hetero biheads with a $[GGGGS]_3$linker in between. In line with the literature (9-11) and taking into account the density of trimer spikes on the virus surface (18), homo biheads based on monoheads with good neutralization properties have to be significantly better than the corresponding monoheads. Indeed, V3A8f homo-bihead neutralize 267 times better than V3A8f, and this mono-head is the best of the selected VHH. The newly selected VHH V2C3 [related to the previously selected CC3] shows a weak neutralization of VEEV-TrD, however a newly selected unrelated VHH like V2B3 neutralizes about a factor 6 better, whereas a homo bihead of this VHH performs

a factor 78 better than the monoheads. Surprisingly a bihead of V2C3 with V3A8f [most likely a hetero bihead] is the best performing one.

Interestingly are the data of neutralization of CHIKV. CC3 is a VHH selected against CHIKV in a previous study (6). A bihead consisting of CC3 and V2C3 or a homo bihead of V2C3 neutralized CHIKV about a factor 8-10 better than monohead. It is worthwhile to test the best mono- and bihead VHH on a wider range of VEEV and on various other members of the genus Alphavirus, like WEEV, EEEV, CHIKV and even on WNV.

At the same time Liu's this paper was published, Bascore et al (18) and Ma et al.(19) published the structure of VEEV in complex with the LDLRAD3 receptor and their results indicate clearly the epitope on VEEV to be blocked. It will be of interest to evaluate with additional experiments and/or with Bio-informatic tools [e.g. Haddock or LZerD (21)] whether the best neutralizing VHH, V3A8f and V2B3, recognize at least part of the epitope on the LDL receptor that is recognized by VEEV. Such analyses may also shed light on why certain VHH raised with VEEV as immunogen also recognize CHIKV [that use MXRAS as receptor] and even WNV. A recent paper of Clark et al. (3) indicates that the Lipid Binding Domain of the group of LDL receptors, including VLDLR and ApoER2 may contain such epitope.

References

1. Sharma S & Knollemann-Ritschel B 2019; Current understanding of the molecular basis of Venezuelan equine encephalitis virus pathogenesis and vaccine development. Viruses 11, 163-195
2. Knipe D.M, & P.M. Howley, In Fields Virology 5th edition, Chapter 31
3. Clark L.E. et al. (2022); VLDLR and ApoER2 are receptors for multiple alphaviruses. Nature 602, 475-480
4. Van Heeke G. et al 2017; Nanobodies as inhaled biotherapeutics for long diseases. Pharmacology & Therapeutics 169, 47-56
5. Jovcevska I & Muyldermans S 2020; Therapeutic potential of Nanobodies. BioDrugs 34 11-26
6. Liu J.L. et al. 2019; Selection and characterization of protective anti-chikungunya virus single domain antibodies Mol. Immunol. 105, 190-197.
7. Liu J.L et al (2022); Bivalent single domain antibody constructs for effective neutralization of Venezuelan equine encephalitis. Science Report doi:org/10.1038/541598
8. Van de Vaart J.M. 2006; Reduction in morbidity of rotavirus induced diarrhoa in mice by yeast produced monovalent lama derived antibody fragments. Vaccines 245, 4130-4137
9. Hultberg A. et al. 2011; Llama derived single domain antibodies to build multivalent superpotent and broadened neutralizing anti-viral molecules. PloS One 6/4/e17885
10. Vanlandschoot P et al. 2011; Nanobodies: New ammunition to battle viruses; Antimicrob. Res 92, 389-408
11. Strokappe NM et al 2019; Super potent bispecific Llama VHH antibodies neutralize HIV via a combination of gp41 and gp120 epitopes. Antibodies 8.38 doi:10.103390/1ntib 8020038
12. De Schacht N et al. 2010; A novel promiscuous class of camelid single domain antibody contributes to antigen

binding repertoire. J. Immunol. 184 doi: 10.4049/jimmunol/0903722

13. Achour I et al 2008; Tetrameric and homodimeric Camelid IgGs originate from same IgH locus. J. Immunol. 181, 2001-2009

14. McCoy LE et al 2014; Molecular evolution of broadly neutralizing Llama antibodies to the CD4 binding site of HIV. PLoS Pathogen 10/12/e1004552

15. Mitchel L.S & Colwell L.J. 2018; Comparative analysis of nanobody sequences and structure data; Proteins Protein doi:10.1002/prot25497

16. Bond C.J. et al 2003; Contribution of CDR3 to VHH domain stability and the design of monobody scaffold from naïve libraries. J. Mol. Biol 332, 643-655

17. Bakker G-J et al. 2019; Thermal stability of single-domain antibodies estimated by molecular dynamics simulations. Protein Science 29 429-438

18. Renisio J.G. et al 2002; Solution structure and backbone dynamics of an antigen free Heavy Chain Variable domain (VHH) for Lama. Protein Structure 47, 546-555

19. K. Basmore et al. (2021); Structure of Venezuelan equine encephalitis virus in complex with the LDLRAD3 receptor. Nature 598, 672-676

20. B. Ma et al (2021); Structure of Venezuelan equine encephalitis virus with its receptor LDLRAD3. Nature 598, 677-681.

21. Christoffer C et al 2021; LZerD Protein-protein docking webserver enhanced with de novo

Table 1 Reconstruction of IMMUNIZATION SCHEMES and IMMUNOGENS USED

Llamas used for immunization

Whisper	**Centavo**	**Cowboy**
Prior imm [8 years]	*Prior imm [8 years]*	*No data on immunization*
PREVENILE + RECOMBITEK [CONT EQUINE VACCINES WNV]	IMM WITH EQUINE VACCINE [WNI + VEWT CON INACT VEEV]	
Imm. scheme not given	Imm. 0, 21, 42, 63 days	
	Prior Immunizations [one year] CHIKV immunogen → Clone CC3	

Immunizations described in this paper

Whisper	**Centavo**	**Cowboy**
WNI + VEWT	WNI + VEWT [EQUINE VACCINE]	WNI + VEWT
Imm days 0, 14, 28, 42	Imm days 0, 14	Imm days 0, 14, 28, 42
Bleed day 56	Bleed day 28	Bleed day 56