Single Domain Antibodies as Medical Countermeasures for Viral Induced Encephalitis

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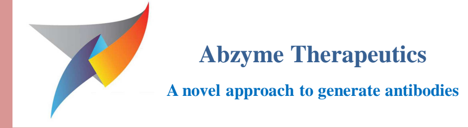

Abstract

Alphaviral equine encephalomyelitis, which results in severe neurological disease and fatalities in horses and humans in the Americas, is caused by Eastern equine encephalomyelitis virus (EEEV), Western equine encephalomyelitis virus (WEEV), or Venezuelan equine encephalomyelitis virus (VEEV). Eastern equine encephalitis (EEE) virus is the most lethal among the three equine alphaviruses. The mortality rate caused by EEEV is 50-75% in humans. The mortality rate caused by WEEV is 3-7% in humans. VEEV is an emerging disease threat with outbreaks first occurring in the 1930s in South America. VEEV infection may cause 1% mortality in humans. There are currently no treatments or vaccines for alphaviral infections available.

The objective of the research was to develop single domain antibodies broadly reactive to alphaviruses that can be used as a countermeasure to viral induced encephalitis using Abzyme's *ex-vivo* so-called ***S****elf-****D****iversifying* ***A****ntibody* ***Lib****rary* or SDAlib generation platform. Our *ex vivo* approach not only shortens time for antibody discovery, but is also applicable for non-immunogenic or toxic antigens and avoids the use of animals. This will allow for quick generation of vaccines to any potential biological warfare agent and the camelid format will simplify storage problems for remote areas.

Camelids produce native homodimeric heavy-chain antibodies, the paratope being composed of a single-variable domain called VHH. VHHs are smaller than conventional antibodies and can recognize antigenic sites that are normally not recognized by conventional antibodies such as enzyme active sites and conserved cryptic epitopes. The affinities of VHHs to antigens are generally comparable to those of conventional antibody fragments with affinity constants (*K*D) as low as 100 pM. In contrast to conventional antibodies, VHHs remain functional after incubation at raised temperatures. Additionally, VHHs are especially suitable as modular building blocks for development and manufacture of multispecific chimeric proteins.

Abzyme's platform is a naive single domain camelid antibody library displayed on the cell surface of highly engineered yeast and is capable of self-diversifying via somatic hypermutation. The platform delivers a new accelerated non-animal approach for rapidly developing high affinity target-specific antibodies. The main advantage of the system is the ability to self-generate a library (and sub-libraries) with diversified antibodies by induced hypermutation in the antibody encoding genes by exploiting three factors for generating hypermutation: (i) an inducible expressed mutator protein; (ii) exposure to an external mutagen and (iii) chromosome polyploidy as a yeast-genome protecting factor allowing the yeast library to be exposed to these mutation inducers for optimum antibody encoding gene diversification.

Using our platform of a self-diversifying camelid antibody library, a set of single domain VHH antibodies broadly reactive to both Eastern and Western equine encephalitis viral E2 glycoproteins have been successfully isolated. One VHH clone (578-S8L1-26) with basic pI (9.3) was identified which should enhance the ability to cross the blood brain barrier. With these research and other projects we have shown that we can quickly develop antibodies against different targets which will be useful for counter- measurements against different future threats.

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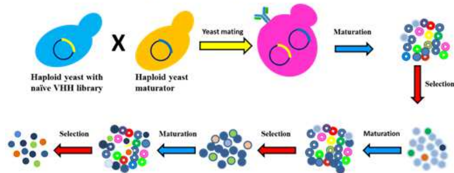
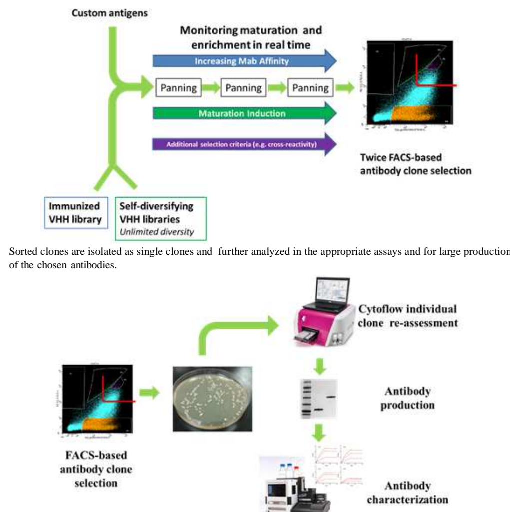
Experimental approach

Our state of the art rapid recombinant monoclonal antibody discovery technologies utilize yeast triple-mode system consisting of *in vivo* antibody maturation, cell surface presentation and secretion. Our systems consist of antibody maturation by inducible *in vivo* mutation without the need to isolate and re-transform DNA into the cells, a surface expression of the antibodies for quick screening and selection of clones with desired attributes adaptable to different requirements (e.g. cross-reactivity of an antibody), and finally inducible secretion of the antibody for a detailed analysis of the selected antibody clone.

Antibody discovery and optimization



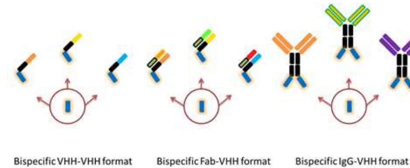
By changing the growth condition, surface expression of the antibodies is induced and the random mutation of the antibody encoding genes is enhanced to achieve an antibody maturation. Target specific clones are isolated by incubation of the cells with labeled antigen and magnetic beads followed by FACS. The cell selection conditions during the panning and sorting steps can be modified to achieve additional specifications of the antibody (e.g. exclusion or inclusion of cross-reactivity).



Sorted clones are isolated as single clones and further analyzed in the appropriate assays and for large production of the chosen antibodies.

Abzyme's modular antibody platform rapidly reformats existing antibody into bispecific antibody

We create bispecific antibodies by combining an active antibody with an active llama VHH at the C-terminus of the antibody. To increase the probability of a reactive VHH in the bispecific antibody, we isolate antigen specific llama VHH from a C-terminal expression library. The llama VHH fragment can be easily shuffled to other antibodies converting them into a bispecific antibody.



Results

We have isolated several encephalitis E3E2 reactive antibody expressing clones based on their results in single clone cytoflow analysis. All isolated clones were sequenced and subdivided into 11 families based on amino acid sequence homologies. Based on our experience with other projects the different families should recognize different epitopes.

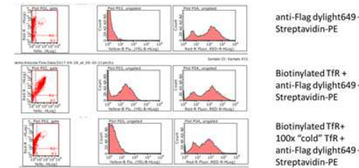
All the clones were subcloned into *E. coli* to produce VHHs and to analyze the binding of the VHHs to E2 protein in an ELISA. For one family (clones 42, 44 and 45) binding to WEEV or EEEV E2 protein could not be confirmed in the ELISA assay. Of the positive clones large protein preparations were prepared to test the binding not only to purified protein but also to inactivated virus particle in an ELISA. The virus particle were prepared by Douglas Reed, the Center for Vaccine Research, University of Pittsburgh. Several of the VHHs react with virus particles of the three different strains (Western, Eastern, and Venezuelan). The reactivity to the three strains differs with the strongest reactions with Eastern encephalitis virus. Comparable amounts of total protein were loaded into each well, however the exact concentrations of E2 proteins of each preparation are not known.

Clone	Reactivity (Target/BSA)		
	Venezuelan	Eastern	Western
17 (20ug/ml)	**2.06**	**6.91**	**3.75**
21 (2ug/ml)	**2.59**	**6.04**	**3.55**
26 (2ug/ml)	**2.18**	**5.20**	**3.40**
66 (20ug/ml)	**1.47**	**4.33**	**2.61**
77 (20ug/ml)	**1.38**	**1.96**	**1.15**

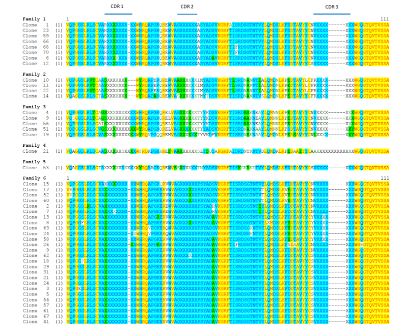
Table 1: ELISA results with VHHs from the different clone families. The supernatants containing VHHs were tested in different concentrations against the different virus preparations and BSA as control. Reactivity ratio was determined by dividing the signal of wells containing virus by the signal of the wells containing BSA. Due to the binding difference between VHHs the data for the dilution with the better response are shown.

De Cogan *et al.* had described that positively charged proteins can cross membranes by themselves (1). Therefore we determined the pI of the virus reactive clones, which ranges from 4.58 (clone 17) to 9.3 (clone 26).

Another proven method to cross the blood barrier method is the use of an anti-TfR antibody as a carrier molecule. (2). To generate bispecific we isolated anti-TfR VHHs. We isolated several clones which were reacting specific with TfR in the single clone cytoflow assay.



The isolated TfR reactive antibody expressing clones we subdivided into 6 families based on their sequence homologies. The pI of the different clones ranges from 8.98 up to 10.02



From each family we selected a few members to produce VHHs in *E. coli* and test their reactivity in ELISA assays.

Clone	Ratio	Clone	Ratio	Clone	Ratio	Clone	Ratio	Clone	Ratio
1	**10.24**	10	**12.33**	56	**11.49**	53	**11.19**	52	**9.40**
68	**9.45**	22	**10.62**	51	**9.32**			13	**8.81**
70	**7.99**							8	**12.10**
								58	**5.12**

Table 2: ELISA results with VHHs for the different clone families. Based on the size of the family different number of clones were selected. The supernatants containing VHHs were tested against human TfR and BSA as control. Reactivity ratio was determined by dividing the signal of wells containing TfR by the signal of the wells containing BSA.

Summary and outlook

We have successfully isolated several VHHs reacting highly specific with either EEV E2 protein or TfR in ELISA assays, by using Abzyme's SDAlib platform . Based on their amino acid sequences of the CDRs we predict that the different anti-E2, as well as the anti-TfR, VHHs recognize different epitopes of their respective targets.

Currently we are establishing the *in vitro* blood brain barrier model in the transwell system to test which of the TfR VHHs has the highest cell layer crossing capability mimicking the blood-brain barrier. For this test we are using hCMEC/D3 cells (3). Additionally, we will analyze in this model if the high pI of some of the anti-E2 VHHs is sufficient to enable the VHHs to penetrate/cross cells (see De Cogan *et al.*). Based on the result we will construct several anti-EEVE2-anti-TfR bispecific antibodies to test their therapeutic potential in *in vitro* as well as in *in vivo* assays. This work will be done in collaboration with Prof. Douglas Reed, University of Pittsburgh.

Literature

1) De Cogan *et al*, " Topical delivery of anti-VEGF drugs to the ocular posterior segment using cell-penetrating peptides", 2017, IOVS **58**:2578
2) Yu *et al*, "Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates", 2014, Science Trans Med **261**:1
3) B. Weksler *et al*, "The hCMEC/D3 cell line as a model of the human blood brain barrier", 2013, Fluids and Barriers of the CNS **10**:16

Support

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