Immunology Letters 188 (2017) 89–95

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Selection of similar single domain antibodies from two immune VHH libraries obtained from two alpacas by using different selection methods

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ARTICLE INFO

Keywords:
Anti GFAP VHH
VH
VHH
Phage display
Ribosome display

ABSTRACT

The two most used methods to select camelid single-domain antibody-fragments (VHHs) are: displaying their repertoires on the surface of filamentous bacteriophages (phage display) or linking them to ribosomes (ribosome display). In this study, we compared specific VHHs isolated from two different immune libraries coming from two different alpacas by using these two selection methods.

Three anti-GFAP (glial fibrillary acidic protein) VHHs were derived from an immune library obtained by ribosome display after immunization of one alpaca with purified GFAP, a protein expressed by astroglial cells. In parallel, three other anti-GFAP VHHs were derived from an immune library by phage display after immunization of another alpaca with a human brain tissue extract containing GFAP. All the VHHs were closely related and one VHH was found to be strictly identical in both studies. This highlights the selection pressure exerted by the camelid immune system to shape the paratope of an antibody against a defined antigen.

1. Introduction

Besides producing conventional tetrameric antibodies, camelids also display functional antibodies composed of homodimerized heavy chains. These antibodies lacking light chains, interact with antigens by the virtue of only one single variable domain of the heavy chain, referred to as VHH to distinguish it from VH of conventional antibodies [1]. VHHs possess a number of advantages for antibody engineering when compared with the conventional antibody fragments (Fab: Fragment antigen-binding or scFv: Single-chain variable fragment). VHHs are highly soluble and can be expressed at high levels in the periplasm and the cytoplasm of *E. coli* and eukaryotic cells [2,3]. Due to their relatively small size (14–17 kDa), VHHs offer also many advantages in terms of structural analysis and in diagnostic applications requiring tissue penetration [2–9]. Achour et al. [10] demonstrated that a single immunoglobulin heavy chain locus in alpaca chromosome 4 contains all of the genetic elements required for generation of the two types of antibodies.

Phage and ribosome display are ideal tools of widespread use for the selection of different antibody fragments [11,12]. Phage display was first described by Smith [13] and McCafferty et al. [14] used it successfully for the selection of antibodies for the first time in 1990. Compared with other display technologies, phage display possesses many advantages: robustness, simplicity and stability of the phage particles and the selection can be performed on cell surface, tissue sections and *in vivo* [15–18]. However, phage-display libraries sizes are restricted to 10^{10}-10^{11} by the efficiency of bacterial transformation. *In vitro* display technologies have since been developed as an alternative, the most widely used being ribosome display [19]. Hanes et al. successfully selected antibody fragments by using this method in 1998 and 2000 [20,21]. Despite many improvements made recently, ribosome display is still challenging to perform for selection on cell surfaces or against non-purified antigens. On the other side, a strong advantage of ribosome display over phage display, is that the encoded DNA does not need to be imported into a host because DNA amplification and protein synthesis occurs totally *in vitro*. As a consequence, ribosome display libraries can allow more diversity with possibly more than 10^{13} binders [12].

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http://dx.doi.org/10.1016/j.imlet.2017.07.001
Received 9 March 2017; Received in revised form 14 June 2017; Accepted 2 July 2017
Available online 06 July 2017

Fig. 1. Sequence of anti-GFAP VHHs obtained by concurrent phage/ribosome display methods. Dotted lines indicate identical sequences. CDRs were determined according to IMGT. * indicated VH hallmarks present in FR2.

Here we wanted to compare VHHs directed against the same protein obtained from two immune libraries by using the two pre-cited most-used selection techniques for antibody screening. The antigen of interest was GFAP (glial fibrillary acidic protein), a well-known marker of astrocytes. Two alpacas were immunized either with a human brain extract that contained GFAP as well as numerous other proteins/peptides, or with purified human GFAP. Then immune libraries were prepared from each animal. Using phage display or ribosome display applied to the two respective different immune libraries, we have selected closely related anti-GFAP VHHs. Unexpectedly one common VHH was isolated from these two different libraries. This emphasizes the strength of selection pressure exerted by the camelid immune system to shape the paratope of an antibody against a defined and highly specific antigen and highlights that selection of a relevant VHH against an epitope is not always dependent on the selection method.

2. Materials and methods

2.1. Immunization

Postmortem brain tissue (hippocampal region) from a patient with Alzheimer's disease was obtained from the NeuroCEB brain bank. This bank is associated to a brain donation program run by a consortium of patient associations and declared to the Ministry of Research and Universities, as requested by French Law. An explicit written consent was obtained for the brain donation in accordance with the French Bioethical Laws. A brain homogenate was performed with this brain sample according to Mercken et al. [22]. Briefly, tissues are homogenized in 10 mM Tris, 1 mM EGTA, 0.8% NaCl, 10% sucrose buffer containing protease and phosphatase inhibitors (Pierce), followed by centrifugation at 27,000*g* for 20 min 1% Beta-mercaptoethanol and 1% N-Laurylsarcosine is added to the supernatant and the mixture is incubated for 2.5 h at 37 °C. After centrifugation at 100,000*g* for 35 min, the pellet is homogeneized in PBS (Phosphate-buffered saline) at a concentration of 1 mg/ml. This human brain extract is referred to as Sg4697. One alpaca was injected with 90 µl of the mixture mixed with 90 µl of Freund complete adjuvant for the first immunization, and with 90 µl of Freund incomplete adjuvant for the following immunizations. Another alpaca was previously immunized with purified human GFAP (USBiological) [8]. In France, an ethical approval is not required for immunization of camelids with Freund (in-) complete adjuvant. The immune responses were monitored by titration of serum samples by ELISA on coated GFAP. The bound alpaca antibodies were detected with polyclonal rabbit anti-alpaca IgGs [23]. The same procedure was performed on a murine brain extract. The extract was referred to as mWT45.

2.2. Library construction, selection and expression of VHH

The blood of the animal immunized with the human brain extract was collected. The selection of cDNA coding only for VHH were realized according to Lafaye et al. [23,24]. The *vhh* genes were then cloned into phagemid vector pHEN1 by using primers contained enzymatic NcoI and NotI restriction sites at the 5′ and 3′ends, respectively. The size of the library was 3×10^8 cfu.

Phage display protocols were performed as described in Lafaye et al. [23]. Briefly10^{13} phage-VHH diluted in PBS were used to perform a panning cycle by using saturated Nunc immunotubes (InterMed) coated with human brain extract (10 µg/ml). To increase the stringency, different blocking buffers for each panning were used: 2% skimmed milk as saturating agent for the first round; 5% BSA (Bovine Serum Albumin) and Odyssey blocking buffer (LI-COR Biosciences) diluted at ¼ for the second and third one.

Sixty-one individual clones were randomly chosen and tested by ELISA against Sg4697 and BSA. The DNA corresponding to the positive VHHs was sequenced by GATC Biotech. The sequences were processed with DNA strider and analyzed using ClustalW2-Multiple Sequence Alignment of EMBL-EBI.

The blood of the animal immunized with GFAP was collected and selection of cDNA coding for VHH was performed as described earlier. The *vhh* population was converted to ribosome display format using PCR and transcribed to mRNA [8]. Selection of ternary complexes involving mRNA, ribosome and functional VHH was performed on GFAP as described by Perruchini et al. [8].

The VHH sequences were analyzed at the genomic level using IMGT (International ImMunoGeneTics information system) alpaca genomic database.

To summarize, the VHHs A12, G110, C5F7, C7G7, B70, B7, G11 and