ELSEVIER

Journal of Immunological Methods 324 (2007) 13–25

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Research paper

Alpaca (*Lama pacos*) as a convenient source of recombinant camelid heavy chain antibodies (VHHs)

David R. Maass [a], Jorge Sepulveda [c], Anton Pernthaner [b], Charles B. Shoemaker [c,*]

[a] *School of Biological Sciences, Victoria University, Wellington, New Zealand*
[b] *Hopkirk Research Institute, AgResearch Grasslands, Palmerston North, New Zealand*
[c] *Department of Biomedical Sciences, Tufts Cummings School of Veterinary Medicine, North Grafton, MA, 01536, United States*

Received 23 February 2007; received in revised form 11 April 2007; accepted 18 April 2007
Available online 15 May 2007

Abstract

Recombinant single domain antibody fragments (VHHs) that derive from the unusual camelid heavy chain only IgG class (HCAbs) have many favourable properties compared with single-chain antibodies prepared from conventional IgG. As a result, VHHs have become widely used as binding reagents and are beginning to show potential as therapeutic agents. To date, the source of VHH genetic material has been camels and llamas despite their large size and limited availability. Here we demonstrate that the smaller, more tractable and widely available alpaca is an excellent source of VHH coding DNA. Alpaca sera IgG consists of about 50% HCAbs, mostly of the short-hinge variety. Sequencing of DNA encoding more than 50 random VHH and hinge domains permitted the design of PCR primers that will amplify virtually all alpaca VHH coding DNAs for phage display library construction. Alpacas were immunized with ovine tumour necrosis factor α (TNFα) and a VHH phage display library was prepared from a lymph node that drains the sites of immunizations and successfully employed in the isolation of VHHs that bind and neutralize ovine TNFα.

Keywords: Recombinant antibody; VHH; HCAb; Camelid; Alpaca; TNF

1. Introduction

The existence in camelids of functional heavy chain IgGs (HCAb) that are devoid of light chains was first demonstrated by Hamers-Casterman et al. (1993). This class of IgG, recently reviewed by De Genst et al. (2006), is fully able to bind to antigens despite the absence of a heavy chain CH1 domain and the inability to combine with light chains. It is thought that HCAbs arose by the loss of a splice consensus signal in the CH1 exon of an ancestral camelid (Nguyen et al., 1999; Woolven et al., 1999) together with compensating amino acid substitutions that improved its hydrodynamic properties in the absence of associated light chain (Hamers-Casterman et al., 1993; Muyldermans et al., 1994; Vu et al., 1997). As a result of the altered splicing, the amino acid sequence that joins the V_H domain to the CH2 domain in HCAbs, called the "hinge" region, is unique to this class of antibodies (Hamers-Casterman et al., 1993). Two distinct hinge sequence types are found in camels and llamas, commonly referred to as the short hinge and the long

Abbreviations: VHH; single-domain antibody fragment; HCAb; heavy chain only antibody; TNF; tumour necrosis factor; PBL; peripheral blood lymphocytes; PCR; polymerase chain reaction; CFU; colony forming unit; HRP; horseradish peroxidase; Ig; immunoglobulin; RACE; rapid amplification of cDNA ends.

* Corresponding author.
E-mail address: charles.shoemaker@tufts.edu (C.B. Shoemaker).

0022-1759/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2007.04.008

hinge (Hamers-Casterman et al., 1993; van der Linden et al., 2000). The V_H region of HCAbs, called VHH, is similar to conventional V_H domains but has unique sequence and structural characteristics (Vu et al., 1997; Harmsen et al., 2000; Decanniere et al., 2000).

HCAbs are able to bind to antigen targets with binding properties that appear equivalent to those achieved by conventional IgG (van der Linden et al., 2000), despite the fact that these antibodies lack the additional antigen contact points normally contributed by light chains. The antigen combining sites of HCAbs thus involve amino acids from only a single VHH domain. DNA encoding this domain can readily be cloned and expressed in microbes to yield high levels of soluble protein that retain the antigen-combining properties of the parent HCAb (Arbabi Ghahroudi et al., 1997). In addition to the small size of these recombinant VHH binding agents, and their ease of production, several other significant advantages have been found. For example, VHHs are generally more stable, particularly to heat (van der Linden et al., 1999; Dumoulin et al., 2002), than conventional antibody fragments and are often found to have unusual epitope specificities, particularly an improved ability to bind active site pockets to produce enzyme inhibition (Lauwereys et al., 1998).

Because of the many favourable properties of VHHs, they have become widely used in research and are beginning to show commercial potential (Gibbs, 2005). Commonly, VHH coding DNAs are amplified from camelid B cell mRNA and a phage library is prepared to display the encoded VHHs. VHHs having the desired antigen binding specificity are then isolated by affinity selection (Arbabi Ghahroudi et al., 1997). Some researchers have obtained VHH agents with desired specificity from non-immune libraries (Verheesen et al., 2006), but immune libraries lead more directly to VHHs with higher affinities (Nguyen et al., 2001).

The source of VHH coding DNA was initially Old World camels (Arbabi Ghahroudi et al., 1997) although these animals are not particularly tractable or widely available. Llamas, which are New World Camelidae, have also been successfully used as the genetic source of VHH clones using PCR primers based mostly on sequence information from camels (Harmsen et al., 2000; van der Linden et al., 2000). In a recent paper, the first use of alpacas, also New World Camelidae, as a source of VHHs has been reported (Rothbauer et al., 2006). This research team, which has pioneered the application of camelid VHHs, stated that alpacas "are the least demanding of all *Camelidae* and alpaca immunization is readily available in most countries". The oligonucleotide primers used to amplify camelid VHHs are generally based on IgG sequences obtained from camels and thus may not be optimal for other camelids and result in the omission of many VHHs from immune libraries. Here we characterize the immunoglobulin component of alpaca sera and report an optimized primer design for PCR amplification of alpaca VHHs based on a representative sampling of random cDNAs. This report should facilitate the utility of alpacas as a genetic source of VHHs.

2. Materials and methods

2.1. Preparation of alpaca lymphocytes

Alpacas were purchased locally and maintained in pasture. All animal experiments were approved by the Wallaceville Animal Ethics Committee. Blood was obtained from the jugular vein and collected into heparinised or serum collection tubes. White blood cells were isolated from about 10 ml of heparinised blood by centrifugation and peripheral blood lymphocytes (PBL) were partially purified by separation over HISTOPAQUE®-1077 (Sigma) using standard procedures and stored in RNA*later* (Ambion). Serum was separated by centrifugation and stored at −20 °C until testing.

The local lymph node from each animal was removed surgically under general anaesthesia, induced with sodium thiopentone (20 mg/kg intravenously) and maintained with halothane (1–3% in oxygen). The prescapular lymph node, which drains the sites of immunizations used in these studies, was removed through a small skin incision and blunt dissection of the fat tissue and muscle overlaying the lymph node. Bleeding was controlled by ligation of the nodal artery and vein. After removal of the node, the edges of the dissected tissue and skin were re-apposed with sutures. Post-surgical care included a single subcutaneous application of antibiotics (400 mg procaine penicillin + 400 mg dihydrostreptomycin sulphate) and an analgesic (flunixin 2 mg/kg). The excised lymph node was cut into 1–2 mm thick slices and either stored in RNA*later*, or immediately subjected to a phenol/chloroform based RNA extraction protocol.

2.2. Alpaca serum immunoglobulin characterization

Alpaca serum was resolved by SDS-PAGE and stained for protein or transferred to PVDF membranes. Filters were probed with HRP labelled anti-llama IgG (H + L) (Bethyl) by standard western blotting methods. The lanes of the stained gel were scanned in a Kodak Image Station 2000RT and the immunoglobulin bands, identified by the western blot, were quantified using Kodak 1D Image Analysis software. Serum was fractionated by