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

David R. Maass^a, Jorge Sepulveda^c, Anton Pernthaler^b, and 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*

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 (TNF α) 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 TNF α .

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 (Hamers-Casterman et al., 1993). This class of IgG, recently reviewed by de Genst et al (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 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. Material 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 mls 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 RNAlater (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 pre-scapular 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 (400mg procaine penicillin + 400mg 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 *RNAlater*, 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 differential absorption on Protein A and Protein G according to Hamers-Casterman et. al. (Hamers-Casterman et al., 1993). Briefly, 2ml of alpaca serum was diluted 2 fold with PBS and adsorbed onto 5ml Protein G Sepharose (Invitrogen). IgG3 was eluted with 0.15M NaCl, 0.58% acetic acid (pH 3.5) after which IgG1 was eluted from the column with 0.1M glycine-HCl pH 2.7. The Protein G unbound fraction was adsorbed onto 5ml Protein A Sepharose (Invitrogen) and the IgG2 fraction eluted with 0.15M NaCl, 0.58% acetic acid (pH 4.5). All fractions were neutralized and protein concentration determined using BCA Assay (Pierce).

2.3. Immunogen preparation and characterization

The full-length coding sequence for ovine tumour necrosis factor alpha (ovTNF α) was amplified by polymerase chain reaction (PCR) from ovine lymphocyte cDNA using primers based on known sequence (Nash et al., 1991). The ovTNF α coding DNA was ligated into AB6-7 (described below) in frame with the leader sequence. The recombinant soluble ovTNF α was expressed and purified by nickel affinity using standard procedures essentially as described in section 2.8 below. The recombinant ovTNF α was shown to have activity in the bioassay described by Flick and Gifford (Flick and Gifford, 1984).

2.4. Alpaca immunization and serum analysis

Two adult male alpacas were given four immunizations at two week intervals, each including six 0.2 ml intra-dermal and sub-cutaneous injections of the immunogen in the pre-scapular region. The immunogen contained a total of ~400 μ g of ovTNF α for each immunization prepared with 13 mg/ml aluminium hydroxide gel (Sigma) as an adjuvant. Serum samples were obtained in weekly intervals and tested for TNF α -specific antibody response by ELISA. Alpaca antibody bound to ovTNF α was detected in the ELISA using antisera from a rabbit immunized with alpaca immunoglobulins (Green et al., 1996).

Some serum samples were assayed for TNF α neutralizing activity in a standard bioassay (Flick and Gifford, 1984) which measures a reduction in TNF α cytotoxicity. Briefly, 100 μ l/well of murine fibroblast L929 cells were seeded in 96-well plates at 1×10^5 cells/ml and incubated overnight. Dilutions of the serum or purified VHH were prepared in RPMI and incubated with serial twofold dilutions of ovine TNF for 30min. After removing of the supernatants of the cultured L929 cells, 100 μ l of the prepared dilutions containing actinomycin D at a final concentration of 1.0 μ g/ml were added to the wells. Then the plates were incubated at 37 °C for 24 h, the supernatants were removed and 0.5% crystal violet in methanol was added and incubated at room temperature for 10 min. Plates were rinsed gently with water, and the optical density (OD) of bound dye was determined at 620 nm.

2.5. Vector construction and bacterial strains

The phagemid vector HQ2-2 (Maass et al, IJP, [in press](#)) was used for preparation of gene III phage display libraries. This vector derives from pCANTAB5E (GE Healthcare) with additional cloning sites inserted and an M13 gene III leader sequence. The E-tag peptide coding DNA and an amber codon are present, in frame, at the fusion between the displayed protein and gene III. To express larger amounts of soluble VHH, the coding DNAs were introduced into the expression vector, AB6-7. This vector is slightly modified from the arabinose promoter vector, pBAD18 (Guzman et al., 1995), to add additional cloning sites, to use the *E. coli* ompF leader, and to fuse inserted DNA with a carboxyl terminal E-tag and hexahistidine coding DNA. Phagemid vector containing heavy chain cDNA was transformed into *Escherichia coli* TG1 cells (Stratagene). Soluble expression was prepared in *E. coli* Rossetagami cells (Novagen) (see below).

2.6. Alpaca cDNA preparation and VHH cloning

RNA later was removed from PBL and lymph node tissue (prepared as above) prior to RNA extraction. Total RNA was separately isolated from PBL and ~25 mgs of lymph node tissue using TRI REAGENT[®]LS (Molecular Research Center, Inc.) according to the manufacturer's protocol. RNA was column-purified using an RNeasy Mini Kit according to the guidelines of the manufacturer (Qiagen) and the yield was calculated in a spectrophotometer at 260 and 280nm. RNA was stored at -80°C. First-strand cDNA synthesis was performed using SuperScript[™]II RNase H⁻ reverse transcriptase (Invitrogen) and poly(A) oligo(dT)12-18 primer to reverse transcribe up to 5µg of total RNA according to the manufacturer's protocol. cDNA was stored at -20 °C until used for PCR.

Two oligonucleotides (AL.CH2, ATGGAGAGGACGTCCTTGGGT and AL.CH2.2 TTCGGGGGAAGAYRAAGAC) were designed to universally prime reverse transcription of mammalian immunoglobulin mRNA templates at conserved sequence motifs representing the codons that encode amino acids 11 to 15.2 and 4 to 10 (IMGT numbering system), respectively, within the CH2 domains (Lefranc et al., 2005). Reverse transcription of alpaca mRNA was performed with the CH2 primers as indicated by the manufacturer for preparing 5'-RACE-Ready cDNA (Clontech). V_H and VHH cDNAs were then amplified by 5'-RACE using the SMART[™] RACE cDNA Amplification kit (Clontech) using the CH2 primers. The two-band product representing VH-CH1-hinge and VHH-CH1-hinge coding sequences was separated by electrophoresis and the lower band (VHH) was cloned into the pCR®2.1-TOPO® vector with the TOPO TA Cloning® Kit (INVITROGEN). Sequencing was performed with the T7 primer.

To prepare VHH phage display libraries, cDNA was first synthesised by reverse transcription from alpaca lymph node RNA using a combination of oligo-dT and pd(N)₆ primers. Superscript II reverse transcriptase (Invitrogen) was incubated with total RNA templates according to the manufacturer's protocol. VHH coding DNA for our early libraries, including the library used to identify anti-ovTNFα VHHs, was amplified from alpaca cDNA using two primer pairs based on those successfully used on llama cDNA (VH1BACK with Lam07 or Lam08 (Harmsen et al., 2000)). The appropriate PCR products corresponding to the VHH were purified via agarose electrophoresis using QIAquick Gel Extraction kit (Qiagen) PCR products were further amplified with primers homologous to the 5' ends of the amplified DNA from the first PCR, and that introduced appropriate restriction sites for cloning into our phage display vector. Later our alpaca VHH libraries employed primer designs based on sequences of random alpaca VHH cDNAs (see Results and Discussion). The primers used were: AlpVh-L (GGTGGTCCTGGCTGC); AlpVh-F1 (GATCGCCGGCCAGKTGCAGCTCGTGGAGTCNNGGNGG); AlpVHH-R1 (GATCACTAGTGGGGTCTTCGCTGTGGTGCG) which primes within the short hinge

coding region at the same site as Lam07; and AlpVHH-R2 (GATCACTAGTTTGTGGTTTGGTGTCTTGGG) which primes within the long hinge coding region at the same site as Lam08.

Amplified VHH DNA was digested with appropriate restriction enzymes and ligated into similarly digested HQ2-2 DNA. The ligated DNA was transfected by electroporation into high efficiency electroporation-competent TG1 cells (Stratagene) following the recommendation of the supplier. Transformants were scraped off the plates and recombinant phage produced according to standard methods (Li and Aitken, 2004). The total number of independent clones present in the library used in this study was 3×10^7 . A quality check was made for each library in which about 40 random clones were picked and PCR amplification was performed using primers flanking the VHH cloning site. An aliquot of each PCR product was analyzed for size by agarose gel electrophoresis. Another aliquot was digested with BstN1 libraries and the “fingerprint” fragment patterns assessed by agarose gel electrophoresis. In libraries used in this study, >95% of the clones had inserts and each of the clones analyzed had unique BstN1 fingerprints (Tomlinson et al., 1992).

2.7. Screening and selection of phage antibodies

Selection was carried out by “panning” of VHH-displayed phage libraries for phage that bind to immunotubes (Nunc) coated overnight at 4° C with 5 µg/ml soluble ovTNF. The tubes were then washed three times with PBS, and blocked with 4% non-fat dried milk in PBS (MPBS) at 37° C for 2 h. A 4 ml suspension of phage in MPBS was prepared containing 5.0×10^{11} CFU was incubated in an immunotube at room temperature for 30 min with continuous rotation, and then for a further 90 min without rotation. The tubes were washed 20 times with PBS containing 0.1% Tween 20 (PBST) followed by 20 times with PBS. Bound phage were eluted by continuous rotation with 1 ml of 100 mM triethanolamine (Sigma) for 10 min, then, recovered and neutralised with 0.2 ml of 1 M Tris-HCl, pH 4.5. A 0.75 ml aliquot of the eluted phage was used to infect 10ml culture of log-phase *E. coli* TG1 cells. A small aliquot of the infected bacteria was used in serial dilutions to titrate the number of phage eluted while the remainder was processed as described above to amplify the phagemid for further selection or analysis. The binding of selected VHHs encoded by phagemid clones to ovTNFα was tested by phage ELISA using anti-M13 antibody (GE Healthcare) for detection. Positive clones were “fingerprinted” by analysis of their BstN1 digestion patterns (Tomlinson et al., 1992).

2.8. Production of soluble single domain antibodies

The VHH coding DNA was subcloned into the expression vector AB6-7 using the same restriction sites as in the HQ2-2 cloning. *E. coli* Rosetta-gami containing the VHH expression plasmid were grown to an optical density of 0.5 at 600 nm and then overnight in 0.1% arabinose at 28° C. Soluble protein was purified from sonicated cells and the recombinant VHH was purified by nickel affinity using Ni-NTA (Qiagen) as recommended by the manufacturer. Protein eluting in 0.2M imidazole was dialyzed against PBS. Purity of the recombinant VHH was assessed by Coomassie Blue staining of SDS-PAGE and protein concentration determined by BCA (Pierce). Western blot and ELISA detection of recombinant VHH was done using HRP anti-E-tag antibodies (GE Healthcare).

2.9. Competitive ELISA

Nunc Maxisorb plates were coated overnight at 4° C with 5 µg/ml soluble ovTNFα in PBS, and then blocked with 4% MPBS. After washing three times with PBS, serially diluted soluble VHH antibodies were added and incubated at room temperature for 90 min to determine saturation curves. To detect bound VHH, an anti-E-Tag/HRP antibody (Amersham) was added at 1:8000 dilution in 4% MPBS for 90 min at room temperature. The wells were washed and developed with 3,3',5,5'-tetramethyl benzidine (Applichem). Competitive ELISA was

performed by preparing plates as described above and then a saturating amount of soluble expressed VHH was added and incubated for 60 min at room temperature. PBS was added to control wells containing no expressed soluble protein. Serial dilutions of bacterial supernatants containing VHH-displaying phage were added for 90 min at room temperature. Plates were then washed three times with PBST and three times with PBS. To detect bound phage, an anti-M13/HRP antibody (Amersham) was added at 1:8000 dilution in 4% MPBS for 90 min at room temperature. The wells were washed and developed as described above. Percent binding was calculated as the $OD_{test}/OD_{control} \times 100$.

2.10. Additive ELISA

Additive ELISA was based on the method of Friguet et. al. (Friguet et al., 1983). Plates were prepared as described above and the dilution of each VHH that able to saturate the coated antigen as determined above was added individually and in pairs. After incubation and washing as described previously, bound antibody was detected by the addition of anti-E-tag/HRP antibody diluted at 1:8000. The additivity index (A.I.) was determine by $A.I. = (2A_{1+2}/A_1 + A_2 - 1) \times 100$ where A_1 , A_2 and A_{1+2} are the absorptions reached, in the ELISA, with the first VHH alone, the second VHH alone and the two VHHs together (Friguet et al., 1983).

3. Results

3.1. Alpaca serum immunoglobulins

Figure 1A shows protein staining of alpaca serum, resolved by SDS-PAGE, from two different alpacas. Figure 1B shows a western blot of the alpaca serum proteins recognized by llama anti-IgG (H+L) antibodies (Bethyl). As has been previously observed for other camelids (Hamers-Casterman et al., 1993) (van der Linden et al., 2000), alpaca serum contains multiple IgG forms. The alpaca serum were subjected to differential absorption (Hamers-Casterman et al., 1993) on Protein A and Protein G and separated into a conventional immunoglobulin fraction (IgG1) and two heavy-chain-only immunoglobulin (HcAb) fractions. Each fraction was characterized by non-reducing (Fig. 1C) and reducing SDS-PAGE analysis (Fig. 1D). As expected, the purified conventional IgG (with two heavy chains and two light chains) in fraction 1 migrates as a single protein of about 175 kDa which dissociates into heavy and light chains of about 50 and 25 kDa under reducing conditions.

Fractions 2 and 3 contain a single protein species of about 90 and 80 kDa under non-reducing conditions that migrate as proteins of about 45 and 40 kDa under reducing conditions. The two HcAb forms fractionate similar to IgG from llama rather than camel with the larger 90 kDa form binding to protein G and the shorter form only to protein A (Hamers-Casterman et al., 1993) (van der Linden et al., 2000). It was thus possible to unambiguously identify each immunoglobulin form in the western blot. The 50 kDa and 25 kDa proteins on the reducing gel are the heavy and light chains of IgG1 and the 45 and 40 kDa proteins are the heavy chains from the IgG3 and IgG2 HcAbs respectively. In llama and alpaca, but the opposite of camel, the IgG3 heavy chains are of the long hinge variety while the IgG2 heavy chains have of the short hinge type (van der Linden et al., 2000). Several isotypes of IgG2 have been suggested for lamoids based on monoclonal antibody analysis although these different forms are not distinguished using the fractionation method (Daley et al., 2005).

The approximate molar ratio of the three immunoglobulin fractions was assessed by scanning the protein stained gel in Figure 1A and quantifying the three immunoglobulin forms. This analysis found the molar ratio of IgG1:IgG2:IgG3 to vary between the two alpacas studied, but to average about 50:30:20. These ratios are similar to those observed for llama except that llama seems to have somewhat less total HcAbs (van der Linden et al., 2000).

3.2. Characterization of random VHH coding DNAs

To design PCR primers that reliably amplify a large percentage of alpaca VHH coding DNAs with minimal bias, we obtained DNA sequences from 24 random VHH cDNAs produced by 5' RACE from a primer site within the CH2 coding DNA. The cDNAs derive from four different alpacas. The sequences encoding the alpaca VHH leader and the framework 1 (FR1) regions are shown in Figure 2A. The leader coding region is highly conserved in all of the alpaca VHH cDNAs and includes a motif of contiguous conserved sequence that was used in the design of a PCR primer (AlpVh-L). The DNA encoding FR1 is also well conserved but the coding DNA has significant differences from the same region in camel VHH coding DNA. In fact, a primer that is commonly used to amplify camel and llama VHHs (VH1BACK) (Frenken et al., 2000) (van der Linden et al., 2000) has several mismatches with alpaca VHHs and would appear to be sub-optimal for PCR priming (Fig. 2A).

With the availability of the new AlpVh-L primer, alpaca V_H and VHH coding DNA could be easily amplified by PCR in combination with two hinge-specific primers (AlpVHH-R1 and AlpVHH-R2). Additional sequences were obtained from the PCR products of these primers using a cDNA template from four different alpaca mRNAs (submitted to Genbank). In total, more than 50 different random alpaca VHH coding sequences were obtained including the FR1 and hinge domains. From this information, a new oligonucleotide pool (AlpVh-F1) was designed to include a primer set that should prime DNA synthesis of most or all alpaca VHH cDNAs from the beginning of the FR1 domain (Fig. 2A). This primer was used to amplify a sampling of diverse VHH cDNAs in combination with the CH2 primer set. The results (Figure 2B) showed that each of the VHH clones amplified efficiently and the products were of similar yield. In contrast, when the VH1BACK primer was used as the FR1 primer with the CH2 primer set, the yields were much more variable and a number of clones failed to amplify. This result suggests that PCR using this primer combination with alpaca cDNA would not amplify a product that accurately represented the VHH repertoire and that a significant percentage of VHHs would be lost.

Table 1 shows the alpaca VHH amino acid sequences extrapolated from the random cDNA clones after removing a few that contained apparent splicing errors and several that were nearly identical (presumably from clonally-related B cells). The VHH protein sequences were compared to the current NCBI GenBank database by BLAST searching and the strongest homology was almost always to llama VHH clones despite the fact that many more camel VHH and other mammalian V_H coding sequences are represented in the database. The strong similarity of alpaca and llama VHHs is consistent with their very close evolutionary relationship (Stanley et al., 1994).

The amino acid sequences of HcAbs generally differ at several positions from those of conventional Igs and the alpaca VHHs also have amino acid sequences at these positions that are characteristic of HcAbs. Most distinctive of HcAbs are the amino acids that occur at the sites in the framework 2 (FR2) region (e.g. positions 42, 49, 50 and 52 (Hamers-Casterman et al., 1993; Harmsen et al., 2000) based on the IMGT numbering system (Lefranc et al., 1999)) at which conventional V_H domains interact with V_L domains. The alpaca VHH amino acids at these positions almost always match those of camel and llama VHHs. Another feature that characterizes HcAbs is their tendency to have larger CDR3 domains than conventional V_Hs, a feature thought to increase the repertoire of antigen combining sites (Muyldermans et al., 1994). These longer CDR3 domains also likely permit improved binding to grooves and pockets, and thus increase the likelihood of their binding to enzyme active sites (Lauwereys et al., 1998). The average size of the alpaca CDR3 domains in our random VHH clones (Table 1) is 17.8 amino acids, which is even longer than the 15 amino acid average CDR3 length observed in dromedary VHHs and the 14.9 average length observed in llama (Vu et al., 1997).

Alignment of the VHH sequences (Table 1) clearly revealed the existence of two classes containing either a long hinge (IgG3) or a short hinge (IgG2) as previously found in other camelid HcAbs (Hamers-Casterman et al., 1993) (van der Linden et al., 2000). About 30% of the random VHH cDNAs encoded the IgG3 class and about 70% the IgG2 class, consistent with the serum characterization data (see above). The alpaca VHH hinge region coding DNA was highly conserved with known camel and llama sequences (data not shown, sequences submitted to GenBank) and confirmed that VHH-specific PCR primers based on known camelid hinge sequences (e.g. Lam 07, Lam 08 (Harmsen et al., 2000) (van der Linden et al., 2000)) should efficiently prime the synthesis of virtually all alpaca VHH mRNAs of the IgG2 and IgG3 classes.

3.3 Alpaca VHH clones that neutralize ovine TNF α

Two alpacas were immunized with biologically active, recombinant ovTNF α which resulted in a high anti-ovTNF α antibody response with endpoint titres exceeding 1:100,000. Serum from the higher responding alpaca at a 1/100 dilution was able to neutralize 4 U of ovine TNF α . During the early immunizations, both alpacas experienced a short term elevation of body temperature, presumably a reaction to ovTNF α which is pyrogenic in sheep. Alpaca sera was separated into IgG1, IgG2 and IgG3 fractions based on differential elution from protein G and protein A (Hamers-Casterman et al., 1993) and assayed for anti-ovTNF α titer. Each IgG fraction contained antibodies that recognized ovTNF α and the titer of each was roughly proportionate to the amount of each antibody (data not shown). The IgG2 fraction was the only fraction in which we could detect a low but significant ovTNF α neutralizing titer.

The immunized alpaca that developed the highest TNF α neutralizing titer also became less sensitive to the pyrogenic effects of ovTNF α injection following several immunizations with recombinant ovTNF α . This alpaca was selected as the source of VHH cDNA for phage display library construction. The lymph node draining the immunization site was surgically removed and V_H and VHH cDNA was amplified from the mRNA with PCR primers to the FR1 and CH2 domains. The product of nested PCR with primers specific to the two VHH hinge regions was ligated into an M13 phage display vector to create a library having a complexity exceeding 10⁷. Phage displaying anti-ovTNF α VHHs were selected by panning and individual positive clones were identified by ELISA. It was necessary to perform only two panning cycles before >50% of the phage clones clearly recognized ovTNF α by ELISA.

Following the panning of the VHH library on recombinant ovTNF α , 72 ELISA positive clones were characterized by DNA fingerprinting. Seven unique anti-ovTNF α VHHs were identified by fingerprinting and DNA sequencing showed them to encode different VHH proteins, although two clones (B5, G7) were very similar and thus appear clonally related. As expected, the anti-ovTNF α VHHs had the same sequence features that were found in the random alpaca VHHs (Fig. 2). The seven VHHs were expressed as *E. coli* soluble recombinant proteins and were obtained in high yield (generally >10 mgs/L of cultured cells). Although each of the VHHs bound ovTNF α , competitive and additive ELISAs (Friguet et al., 1983) both suggested that three of the unique clones bound to the same or overlapping epitope while the other four clones recognized distinct epitopes (data not shown). The anti-ovTNF α recombinant VHHs were individually tested for their ability to neutralize the biological activity of ovTNF α (Figure 3). The same three anti-ovTNF α VHHs that appeared to share an epitope also were the only clones that displayed potent ovTNF α neutralizing ability in a cell based assay. Furthermore, each of the neutralizing VHHs were of the short hinge type while only 1 of the 4 non-neutralizing VHHs were of this type. This is consistent with the finding that the IgG2 fraction of the immune alpaca contained most or all of the neutralizing activity. Interestingly, the neutralizing activity of the three VHHs was highly specific for ovine TNF α . None of the VHHs

was able to neutralize alone or when combined, bovine, human, mouse or possum TNF α (data not shown).

4. Discussion

Camelid VHHs are widely known to have highly favourable properties as antigen binding agents for research and commercial applications, but the poor accessibility, large size, expense and difficult handling that characterizes the use of camels and llamas has significantly limited their general use by scientists. Alpacas are more widely available, less expensive to maintain, smaller and more tractable than camels and llamas and their use as a source of immune B cells for VHH library construction could expand the accessibility of VHHs to many more laboratories. In a recent publication, the first reporting the use of alpacas as a VHH source (Rothbauer et al., 2006), the authors commented on the distinct utility of the alpaca model. Here we show that alpacas appear to be at least as useful as camels and llamas as a source for immune HcAbs and we provide the information necessary to guide researchers in the preparation of VHH libraries that accurately represent the VHH repertoire of the animal.

More than 50 random alpaca HcAb cDNAs were sequenced through the entire VHH coding region to aid in the design of PCR primers. The goal is to use primers that amplify the vast majority of alpaca VHH coding DNAs to produce a DNA pool that closely represents the VHH repertoire of an immunized animal. A highly complex library created from this pool will thus contain a more diverse variety of VHH clones with the ability to bind the immunogen. Obviously, the larger the number of different VHH clones that bind the target of interest will increase the likelihood of finding a VHH with the specific properties that are most important to the researcher. The type of desirable properties being sought might include high affinity, target neutralization, target specificity, stability, high level of functional expression in *E. coli* and others. We were able to design a single primer pool (AlpVh-F1) homologous to the beginning of the FR1 domain that consistently amplified 23/23 diverse alpaca VHH cDNAs in combination with a primer pool homologous to the CH2 domain. Similar PCR using an FR1 primer commonly used to amplify camel and llama VHHs efficiently amplified only about half of the cDNA and failed to amplify several clones. Since llamas are much more closely related to alpacas than to camels (Stanley et al., 1994), it seems likely that VHH primers designed from alpaca cDNAs will improve the quality of VHH libraries prepared from llama B cell cDNA.

Sequencing of the entire VHH leader sequence coding DNA for 25 random cDNAs demonstrated that a primer can be designed from this region as a means to “pre-amplify” the VHH and VH coding DNA prior to amplification of the DNA that will be used for library construction. This would be of distinct value when only a limited amount of B cell cDNA is available. In that case, the cDNA can first be amplified with a combination of a leader sequence primer and a CH2 primer to generate a product of about 600 bp. This DNA can be purified and then used as the template to amplify the VHH coding DNA for phage display library construction. When B cell cDNA is not limiting, the preferred strategy is to directly amplify the cDNA in two separate reactions with Alp-Vh-F1 forward primer in combination with a reverse primer that is specific to either the short hinge or the long hinge to produce a product of about 400 bp for library construction.

For this study, we used tissue from the lymph node draining the immunization site as the source of cDNA synthesis for a VHH library. Lymph node tissue is not difficult to obtain and is expected to yield cDNA that is more enriched in immunogen-specific VHHs than peripheral blood lymphocytes (Basalp and Yucel, 2003), although either source can be used for VHH library construction (Saerens et al., 2004). The ease with which sufficient B cells can be

obtained from camelids makes it readily feasible to use these animals successively as a source of VHHs for multiple immunogens.

As a demonstration of the utility of the immunized alpaca as a source of VHHs against an antigen, we prepared neutralizing VHHs against ovine TNF α . Tumour necrosis factor (TNF) plays critical roles in the initiation, maintenance and resolution of various immune responses. In particular, its overproduction has been implicated in inflammatory diseases such as multiple sclerosis, rheumatoid arthritis and Crohn's disease (Ruuls and Sedgwick, 1999). Anti-TNF therapy can ameliorate much of the pathology from a number of intestinal diseases in humans and animals (Abuzakouk et al., 2002) (Marini et al., 2003) (Lawrence et al., 1998) (Liesenfeld et al., 1999). In the sheep, expression of TNF α is associated with increased chronic intestinal inflammation from parasite infection (Pernthaner et al., 2005). Based on the success of human anti-TNF α therapies, we hypothesize that neutralization of TNF α will diminish the intestinal pathology associated with nematode infection, Johne's and other diseases. Availability of anti-ovTNF α VHHs that can be produced economically at large scale, as reported here, will allow its testing as a therapeutic agent in sheep.

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Abbreviations

VHH	single-domain antibody fragment
HCAb	heavy chain only antibody
TNF	tumor 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

Figure 1. Characterization of alpaca serum immunoglobulins

A. Coomassie blue stained gel following SDS-PAGE of serum from two different normal alpacas under reducing conditions. MW markers are shown (M) and their sizes indicated. B. Western blot of the alpaca serum resolved in A and probed with anti-llama IgG (H+L). The identities of the stained proteins are indicated. C and D. Coomassie blue stained gel following SDS-PAGE of three purified alpaca IgG isotypes resolved under nonreducing (Panel C; 7.5% gel) and reducing conditions (Panel D; 10% gel).

Figure 2. Amino terminal coding sequence of VHH cDNAs and PCR primer design

A. The cDNA encoding the complete amino terminus of 24 random VHH proteins obtained by 5'RACE from normal alpacas. The sequence of the leader and the beginning of the first framework domain are shown. The asterisks below the alignment indicate the positions of 100% nucleotide conservation. Two PCR primers (AlpVh-L and AlpVh-F1) are shown that were designed for amplification of VHH coding DNA based on the alpaca cDNA sequences. PCR primer VH1BACK, which has often been used for PCR amplification of camel and llama VHH coding DNA, is also shown. B. Agarose gel of the PCR amplification products of 23 diverse alpaca VHH cDNAs. PCRs were performed together, each with a single unique cDNA clone as the template. Each reaction contained a mixture of two CH2 'reverse' primers (AlpVHH-R1 and AlpVHH-R2) with either AlpVh-F1 or VH1BACK as the 'forward' primer.

Figure 3. Neutralization of ovTNFα by seven different alpaca anti-ovTNFα VHHs

The assay was performed as described in Material and Methods. Negative (non-immune) and positive (ovTNFα) alpaca serum was used at 1:100 dilution. Each ovTNFα VHHs was tested at 1 μg/ml concentration. G7, F4 and B5 (closed symbols) showed ovTNFα neutralizing activity while C4, C3, D3 and F1 (open symbols) had no significant neutralizing activity. Data were verified in two additional independent experiments.

Table 1
Amino acid sequences encoded by 42 random alpaca VHH cDNA clones separated into predicted protein domain regions

CLONE	LEADER	FR1	CDR1	FR2	CDR2	FR3
A02	VVLAALL	QGIKARQFVESGGGLVQPGGSLRLSCTAS	G--FSLKYWA	VGWFRQAPGK---REGVLC	VSQ-SGE--IT	NVANSVKGRFTISRDNDK
A52	VVLAALL	QGVQAOLQLVESGGGLVQPGGSLQLACEAS	G--VTLFEVS	VAWFRQAPGK---REGVSC	INS-NGNGST	TYARSVKGRFTISRDSA
A53	VVLAALL	QSVDAYLRIVESGGGLVQSGGSLRLSCSAS	G--DKLARYA	VGWFRQAPGK---QREAVAC	IDN-SVG--NT	TYADAVKGRFTISRDKAE
A07	VVLAALL	QGVQAEVLVESGGGSLVQAGGSLRLSCAVS	G--SLASMNS	MGWYRQPSPEK---EREFVAS	INR-SN---YT	TYSTSVKDRFLSRDHDK
A31	VVLAALL	RGVQAEVRLVESGGGLVQAGGSLRLVCVAY	G--TIESYNA	MGWYRQPPGK---ERELVAG	ITA-QG---TL	EYAEAVKGRATISRDNAK
A54	VVLAALL	QGVQAQVPLEESGGALVQPGDSLRLTCKVS	G--NIADVYA	MAWYRQAPGK---QRKMVAG	ISD-AG---TT	NYGTSMRGRVTTISRNSAK
A55	VVLAALL	QGVQAQQLVESGGGLVQSGGSLRLSCEVS	G--TIESIYA	MAWYRQAPGK---QRDLVAA	ITT-TG---TT	DYADSVTCGRFTISRDNAK
A56	VVLAALL	QGVQAQEFQVETGGGLVQPGGSLTLCVY	G-GAVYHRSG	LGWYRQAPGK---ERAWVGR	IHS-G-G--GT	GYADSVKGRFTLSRPFGN
A57	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCAAS	G-FTIENYH-	VRYYRQAPGK---EGELVAG	IAV-GNA--WT	SYADSVKGRFTISRDSN
A58	VVLAALL	QGVRAQVQFVESGGGEVTRGESLRISCLAS	G-GVTSLSY	LAWYRQTPGN---QRETVAV	IKS-G-G--ST	NYADSVKGRFTVSRDNTK
A59	VVLAALL	QGVQGVQFVESGGDLVQPGGSXRLSCEAS	G--IRKLYP	MGWYRQAPGR---ORELVAR	VT--IRE--ST	NYTDSVKGRFTISRDDAK
A60	VVLAALL	QGVQAQQLVESGGGFVQPGGSLRLSCTAS	G--SNLRLYG	MGWFRQAPGK---ERELVAG	ISW-IGG--AA	YYSTSVKGRFTLTRDYDQ
A61	VVLAALL	QGVQAQQLVESGGGFVQPGGSLRLSCTAS	G--STIKNYD	MGWFRQAPGK---EREFVAV	ISG-G-G--GD	YYSDSVKGRFTISRDDGQ
A08	VVLAALL	QGVQATQLVESGGGLVQPGSLRLSCTAS	G--STFRVYK	VGWFRQAPGK---EREKVC	LS---AVE--TT	DYAAAARKGRFTTFRNKAE
A23	VVLAALL	QGVQAQQLHVESGGGSLVQPGGSLRLTCAAS	T--FTLNYS	IAWFRQAPGK---REGVSC	MS---GGG-VT	VYAESVKGRFTISRDSA
A38	VVLAALL	QGVQAQQLVESGGGFVQPGGSLTLCVGS	G--ISLDHRG	IGWFRQAPGK---REGVSC	IN---SDRIT	SYADSVNCRFTISKDRAK
A06	VVLAALQ	QGVQAQQLVAESGGGLVQPGGSLRLSCVAS	G--LTLDRHN	ILWFRQAPGK---DREGVSC	IK---MRGSA	KYADSVKGRFTVSRDNAE
A51	VVLAALL	QGVQAQVHLVESGGGLVQPGDSLRLSCTAS	G--IPLRSHV	IGWFRQVPGK---EREAVAC	ID---GSGGT	NYAGSVLDRFSISRDDAK
A11	VVLAALL	QGVRPQVPRVESGGGSLVQPGGSLRLSCTOP	K--VNLNNFA	IGWFRQAPGK---REGVSC	I---GNRGTP	YYADLEGRFTISRDNAK
A62	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCTAS	D--AAEFYA	IGWFRQAPGK---REGVSC	IS---PSRAFT	NYTDAVKGRFTISRDSK
A43	VVLAALL	QGVQAQQLVESGGGVQPGGSLRLSCTAS	G--GTLDFYA	IGWFRQVPGK---DREGIAC	IS---SGRSS	DYVDEVKGRFTISRDNAK
A47	VVLAALL	QGVQAQQLVESGGGDSVQPGGSLRLSCVAS	G--LNFHDLA	MGWFRQAPGK---EREAVSC	IS---NRGGHT	EYVDSVKGRFTISRDNAK
A39	VVLAALL	QGVQOTQLVESGGGLVQPGGSLRLSCVVS	G--IKLDHYA	FGWFRQAPGK---EREAVGC	IG---SGGRIT	NYRDSVGRGATVSDITGR
A45	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCTVS	G--FALDYYV	IGWFRQAPGR---ERHILSC	IS---SGGST	NYADSVKGRFTISRDNDQ
A03	VVLAALL	QGVQPOVLVESGGGLVQPGGSLRLSCTVS	G--RTLSTYV	IGWFRQAPGK---REGVSS	IS---STDGST	YXSESVKGRFTISRDNAE
A12	VVLAALL	QGVRAQVKLVESGGGFVDAGGSLTLCASS	G--FVLEIFV	IGWFRQVPGK---EREGISC	IS---IRDGSA	YYEDSVKGRFTVSRDNAE
A63	VVLAALL	QGVQPO--OLKESGGGLVRPGGSLRLSCALS	--ERRLEDYA	IAWIRQAPGK---DHEAISC	IS---VGRST	EYSNSVKGRFTVSRDDAR
A64	VVLAALL	QGVQAQVQVAESGGGLVQPGGSLTLCVVT	GTSSLDYIP	VAWFRQVPGK---EREAGISC	IGGVPHGEDHIL	FYSTPVKGRFTSSRDDAK
A30	VVLAALL	QGVQSOQLVETGGGVSPGGSLRLSCTHS	G--FPLERM	ISWFRHRPGKNGDEREGLSC	IS-TIH--GGT	EYADSVKGRFTISRDIAK
A41	VVLAALL	QVVQARVLVESGGGLVQPGGSLRLSCTAS	G--FTFANYA	LNWYRQPPGK---GLEWVSR	----IHSDGDT	KYADSVKGRFTISRDIAN
A44	VVLAALL	QGVQAEVQVAESGGGLVQPGGSLRLSCRAS	G--FTESSYS	MGWARQIPGK---GLEWVSS	----IYSDGST	YYTDSVKDRFILSRDNAK
A65	VVLAALL	LGVQAEVLRESGGGSLVQAGGSLRLSCEAS	G--TTSRIDV	LAWYRQTPGN---QRVFEVAS	IT---RDNGYT	KYADFNCRHDIRDNNAA
A10	VVLAALL	QGVQAQVQLVESGGGLVQPGGSLRLSCVYS	G--ILATGWP	MSWYRQAPLK---GPEWLSD	IN---DG--AT	YYADSVKGRFTISRDDAK
A32	VVLAALL	QGVQAQHLVVESGGGLVQPGGSLRLSCTAS	G--FTLDSHH	IGWFRQAPGK---ERELVSC	TG---TTGDFP	HYADSVKGRFTISRDNAK
A66	VVLAALL	QGVPAQLHVVESGGGLVQPGGSLRLSCKVS	G--LDLDLYL	LGWFRQAPGK---EREWVSC	VD---HSGDLE	VYGDSVGRGFAISRDNK
A36	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCTAS	G--SSLDSYA	IGWFRQAPGK---REGVSC	IN---SSGGIT	NYADSVKGRFTISRDNAK
A67	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCTAS	G--SSLDSYA	IGWFRQAPGK---REGVSC	IN---SSGGIT	NYADSVKGRFTISRDNAK
A48	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCAGS	G--FTLDSYA	IGWFRQAPGK---REGVSC	IS---SSGGST	NYADSVKGRFTISRDNAK
A68	VVLAALL	QGVQARLHLVESGGGSLVQPGGSLRLSCVAS	G--EPLENNA	VGWFRQSPGN---SREGISC	ISTLGSRGITD	DYAGSVKGRFTVSRNDK
A69	VVLAALL	QGVQAQVEPVESGGGLVQPGGSLRLSCTSH	I--EALYHYA	VGWFRQVPGK---KREWVAC	IS---SSGENV	DYHESVKGRFTISKDSTR
A14	VVLAALL	QGVQAQLOPARAGNVLQPGGSLRLFCGVS	E--RTLNGYD	IGWFRQAYGT---EREGISC	TS---RSGNT	VYADSVKGRFTVARDNAK
A17	VVLAALL	QGVQAQQLVESGGGLVQPGGSLRLSCVAS	G--SSLSTYH	IGWFRQAPGK---GREAVAC	IS---DSGGSI	TYADSVKGRFTISRDDAE
CLONE	FR3	CDR3	FR4	HINGE		
A02	NTVYLQMNLSLKPEDTSVYYC	G-DAPTC-----SDSVGDFGS	WGQGTQVLT-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		
A52	NAVYLOMNSLKSEDTAVYYC	SVVEPNC-----SSGRKAFGS	WGQGTQVLT-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		
A53	NAVYLOMNSLKPEDTAIYYC	AADAIYC-----RSP--DFGA	WNQGTQVLT-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		
A07	NILSLOMNGLKPEDTAMYYC	AAEGR-----GWG----PV	LQGGTQVLT-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		
A31	-KLSLQNLNKDEDTAMYYC	AAWDRSDY-----RGHH----DF	LQGGTQVLT-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		
A54	NTVYLDNMNLSLKPEDTAVYYC	NALERTYS-----GGDDYGR--DY	WGKGALVN-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		
A55	NTVYLQMNLSLKPEDTGVYYC	AADGLRLD-----ACVFPRRTYSY	WGQGTQVLT-VSS	-----AHHSDDPS--SKCPKCPGPELLGGP		

CLONE	LEADER	FRI	CDRI	FR2	CDR2	FR3
A56	NTVFLQMNINKPEDTAVYYC	NT-----WPMKAST	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A57	KTVYLQMKSLQPEDTAVYHC	NV-----RVFSAPC	AGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A58	NTMYLQMNLIKPEDTAVYFC	NFPAY---GYGGOVVAQVP	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A59	NMVYLQMTMQPEDTAVYYC	NADLG-----LPSMRFPAP	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A60	KITLLQMNKLSPEDTAVYFC	AAST-----TPPIFTFNS	YGPQTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A61	NTVYLQMNLIKPEDTAVYYC	SIESDPDA-----IGEMIRGKHRGS	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A08	TTYLQMNLIKPEDTAVYYC	AAYYGG---Y-HCLA---BEHYDH	WGQGTQVT-VSA		G-----AHHSEDPs--SKCPKCPGPELLGGP	
A23	NTMYLEMNSLEPEDTAEYYC	AADEEA-----V-CSVATLKRRAKYDW	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A38	NTVYLQMNKPKPEDTGVVRC	AAFTWI---CS-SAYAWANYDMDH--	WGRGTPVN-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A06	KTVYLQMNLSLPDIDTAVYTC	AAEELWN---LSD-GELYPCSPRMEY--	WGKGTILVN-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A51	NTVLLQMNLIKIEDTAVYYC	AAELWN---LSD-GELYPCSPRMEY--	WGKGTILVN-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A11	NTVYLQMNLIKPEDTGVVSC	AADLGAGGCVLSP-AGTLSIARFLEV--	MGPGTILVN-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A62	NTVYLQMTSLTPEDTAVYYC	AADRGG-----PSWCNNGMDY--	WGKGTILVN-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A43	NTVFLQMNLSLSDSDTATYYC	AATLSG-----Y-SGLFWCVRPNSY--	WGQGTQVAVVSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A47	NTVYLQMNLIKQVEDTAVYYC	AAP-----TALRWQCNPAYFGS	WGQGTQV-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A39	NTVHLOMSSLELEDIGLYYC	AA-----VLSY-GSACVISRSDVYOH	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A45	NMAYLEMTGLKPEDTAVYYC	AADQR---LTSLSITCVLDHA--YEF	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A03	NTVYLQMNLIKSEDTAAYYC	AAADPWK-----ISXSTMISOEPYFY	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A12	NTVYLQMNLIKPEDTGVYTC	AAESFWV-----GPVOAMCNP--HI	WGQGTQVT-VSA		-AHHSEDPs--SKCPKCPGPELLGGP	
A63	NMVYLDMNALKPEDTAVYRC	AADSGVR---RHOLCQIDTKRYDY	WGLGTQVT-VPS		-AHHSEDPs--SKCPKCPGPELLGGP	
A64	NTVYLHMNRLTPGDTAVYYC	SPAN-----KGFCITLDANDYKY	WGQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A30	RTVYLQMNLETDSDGVYYC	AVGGEF-----GS-ACPLWGN-MDS--	WGKGAELV-VRS		-AHHSEDPs--SKCPKCPGPELLGGP	
A41	STLYLQMNLIKPEDTGVYYC	VKPGS-----CLYVNAWKY	RGEQGTQVT-VSS		-AHHSEDPs--SKCPKCPGPELLGGP	
A44	SMVYLQMNLIKPEDTAVYYC	TTDLS-IHPESDS-----PPLGSSOHHY	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A65	NTVSLQMNLIKPEDTGYVC	NAHLGRIFPSRDH-----VPWDRADHY-	WGVGIPVT-VSA		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A10	NTLYLQMNLIKPEDTAVYYC	AOPRD--TQVDR-----NSWGR--YVN	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A32	NTVYLQMNLIKPEDTAVYYC	A-ADSGRSWSL-----CPRGSVGYDY	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A66	NTVYLQMNRLPKDOAVYYC	A-ADP---AREFT---CSPD--EYDY	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A36	NTVYLQMNLIKPEDTAVYYC	A-AAPGLTTFQTL-----CLMIGG--DY	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A67	NTVYLQMNLSLKLEDIGIYVC	A-AGSGSTYYDCS-----GYETGWRIDK	WDOGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A48	NTVYLQMNLIKSEDTAVYYC	A-AALGMTTVQNM-----CLMWGSG-NY	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A68	NTVYLQMNLIKPEDTAVYFC	AAVYTGAGOLLPS-----LCLDENGYYD	WGQGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPELXXXX	
A69	NTAYLDIMNIEPEDTATYYC	G--AASDLWYSGL-----YCGP--DYDY	WGQGTQV-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A14	TATFLEMSDLKPEDTGYFC	AALGGDVCVREGRPGAYYCIANRCYDDY	WDOGTQVT-VSS		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	
A17	NTVHLOMTSLKAEDTAVYYC	APVITPG--YYGGS-----YSCIPDGV	WGQGTQVT-VSV		-EPKTPKPOPOPOPOPNPTTESKCPKCPAPPELLGGP	