Generation of a Family-specific Phage Library of Llama Single Chain Antibody Fragments That Neutralize HIV-1*

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Recently, we described llama antibody fragments (VHH) that can neutralize human immunodeficiency virus, type 1 (HIV-1). These VHH were obtained after selective elution of phages carrying an immune library raised against gp120 of HIV-1 subtype B/C CN54 with soluble CD4. We describe here a new, family-specific approach to obtain the largest possible diversity of related VHH that compete with soluble CD4 for binding to the HIV-1 envelope glycoprotein. The creation of this family-specific library of homologous VHH has enabled us to isolate phages carrying similar nucleotide sequences as the parental VHH. These VHH displayed varying binding affinities and neutralization phenotypes to a panel of different strains and subtypes of HIV-1. Sequence analysis of the homologs showed that the C-terminal three amino acids of the CDR3 loop were crucial in determining the specificity of these VHH for different subtype C HIV-1 strains. There was a positive correlation between affinity of VHH binding to gp120 of HIV-1 IIBB and the breadth of neutralization of diverse HIV-1 envelopes. The family-specific approach has therefore allowed us to better understand the interaction of the CD4-binding site antibodies with virus strain specificity and has potential use for the bioengineering of antibodies and HIV-1 vaccine development.

Neutralizing antibodies (NAbs)4 are an important defense mechanism against virus infections and are the basis for many successful vaccines developed against viruses. In HIV-1-infected patients, NAbs exert a selective effect on virus evolution (1–3). NAbs of HIV-1 target the trimeric envelope (Env) glycoproteins, gp120 and gp41, which mediate binding to the primary receptor, CD4 (4, 5), and coreceptor, either CCR5 or CXCR4 (6–11), for virus entry into host cells. However, the elicitation of broad NAbs to HIV-1 in vivo is rare due to the enormous global diversity of the HIV-1 Env and due to protection by carbohydrate moieties of neutralization-sensitive epitopes (12, 13). Most neutralizing monoclonal antibodies (mAbs) have been isolated from humans naturally infected with HIV-1 and are usually derived from patients with long term infection (14). The difficulty in eliciting broad NAbs is also demonstrated in immunization programs in humans or animals with HIV-1 Env-based immunogens and represents a major hurdle in the development of an effective humorally based vaccine against HIV-1.

Despite these hurdles, passive immunizations with NAbs have been shown to prevent infection and the onset of disease in the macaque model (15–18) and to help in the control of disease progression when introduced post-infection (19). These studies show the importance of further investigation of NAbs for vaccine development and disease control. Identification and characterization of novel broad NAbs may provide additional insights into conserved epitopes that may be targeted for the development of vaccines and entry inhibitors. The engineering of antibodies is therefore an appropriate tool for this purpose.

A small number of broadly neutralizing mAbs have been characterized, which recognize epitopes in the membrane-proximal region of gp41 (20–23), the CD4bs (24, 25), the V3 loop of gp120 (25), and a conformational site near the base of the V1/V2 and V3 loops (26). Because the CD4-binding site (CD4bs) must retain some conserved determinants to mediate CD4 binding, NAbs targeting this epitope have the potential to neutralize diverse subtypes of HIV-1. This is confirmed by recent reports that demonstrated the neutralizing activity of broadly neutralizing human sera to be mediated by Abs directed at the CD4bs of gp120 (27–29). We have recently described two llama heavy chain fragment antibodies (VHH), termed A12 and D7, that compete with sCD4 for Env binding and are able to neutralize different subtypes of HIV-1 (30). To our knowledge, this was the first reported instance where potent cross-subtype neutralizing mAbs against HIV-1 were generated from an immunized animal.

Broad NAbs have generally been obtained from natural human HIV-1 infection, after prolonged persistent virus replication and mutation, permitting antibody maturation against multiple conserved epitopes (28). However, the unique proper-

* The abbreviations used are: NAb, neutralizing antibody; HIV-1, human immunodeficiency virus, type 1; Env, envelope; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; CDR, complementarity determining region; CD4bs, CD4-binding site; sCD4, soluble CD4.

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ties of llama antibodies allow the isolation of relatively broad Nabs following immunization with Env antigens, giving rise to the A12 and D7 VHH (30) and other VHH from new immunizations still being characterized. The VHH domain consists of the variable region of the heavy chain antibody, which is a unique form of antibodies that members of the Camelidae family produce (31), and they possess all the antigen-binding properties of the complete antibody. Favorable characteristics of VHH that make them suitable for further investigation as mAbs against HIV-1 include their tendency to have affinity and specificity similar to conventional antibodies (32), their longer complementarity-determining regions (CDRs) (33), and their preference for cleft recognition and binding to active sites (34, 35), as well as the potential to format these in multispecific or multivalent constructs (36). Libraries of single chain antibodies are easily produced and have been successfully used for panning against various pathogens (37–39). Here, we describe a novel method to create a library of related VHH clones that share similar properties to the parental A12/D7 VHH and recognize a similar epitope. The lymphocyte pools from the original immunized llama were mined for VHH variants that were able to recognize the CD4bs of gp120 using molecular techniques, without specific strategies for eluting out CD4bs-specific VHH. A panel of 49 unique VHH were selected from the subfamily library, of which 31 were further characterized.

**EXPERIMENTAL PROCEDURES**

**Recombinant gp120 Antigen Preparation**—Recombinant gp120 from HIV-1 IIIB (catalog no. EVA607) was obtained from the Centralized Facility for AIDS Reagents (National Institute for Biological Standards and Control, Potters Bar, UK). A subtype-C recombinant gp120 from HIV-1 92BR025 was expressed in a mammalian cell culture system and purified as described previously (40). Briefly, the envelope gene was amplified by PCR from proviral DNA and cloned into an expression vector with the incorporation of a C-terminal His$_6$ tag. Recombinant 92BR025gp120 was expressed in 293T cells infected with a T7 RNA polymerase recombinant vaccinia virus (vT7-3, American Tissue Culture Collection number VR-2153). Envelope proteins were harvested 72 h post-transfection and purified using TALON metal affinity resin (Clontech) according to the manufacturer’s instructions. Both of these recombinant proteins were then biotinylated using the EZ-Link biotinylation kit (Pierce), according to the manufacturer’s instructions. The biotinylated recombinant gp120 was dialyzed with phosphate-buffered saline using Microcon (Millipore) with a 50-kDa cut-off, and the integrity of the biotinylated proteins was verified by ELISA and Western blots.

**A12/D7 Family-specific Phage Library Construction**—Total RNA (60 μg) previously isolated from the peripheral blood lymphocyte pools of a llama immunized with recombinant gp120 derived from HIV-1 CN54 was reverse-transcribed into cDNA using random hexamers (SuperScript III, Invitrogen) and cleaned up with QIAquick PCR purification kit (Qiagen). Based on the nucleotide sequences of the A12 and D7 VHH, a unique degenerate reverse primer that extended into the last six codons of the CDR3 loop region was designed to pull out VHH gene fragments with similar sequences through PCR in conjunction with a framework 1-specific primer. The sequences of the A12 and D7 VHH, together with the sequence of the A12/D7 specific primer, are shown in Fig. 1. The A12/D7 CDR3 sequences do not share any homology with other known CD4bs-specific antibodies using a BLAST search. The PCR amplification was carried out with Expand High Fidelity (Roche Applied Science) using the following program: 94 °C for 2 min, followed by 28 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 7 min. The PCR was limited to 28 cycles to prevent oversaturation of the amplified products, and a 350-bp band was excised after separation on an agarose gel. Following restriction enzyme digestion with BstEII and SfiI and gel purification, the digested DNA fragments were ligated into a phagemid vector (pAX50) for display on filamentous bacteriophage and electrotransformed into *Escherichia coli* TG1 competent cells as described previously (41). The transfected cells were titrated on agar plates to determine the library size, and a colony PCR was performed on a selection of colonies to determine the presence of DNA inserts in the vector.

**Bio-panning and Retrieval of Binders**—The family-specific phage library displaying the cloned VHH repertoire was preincubated with four dilutions (0.01–10 nM) of biotinylated recombinant gp120 antigens in 0.2% casein on a preblocked microtiter plate for 2 h. The binding of phages to antigens in solution reduced the avidity effects of the binding. Phages bound to the biotinylated antigens were subsequently captured by incubating the mixture for 30 min on immobilized neutra- din (Sigma) or on D7324 (Aalto Bio Reagents, Dublin, Ireland) that was precoated overnight in 96-well Maxisorp plates (Nalgene, Hereford, UK). D7324 is a sheep polyclonal antibody raised against a conserved motif in the C terminus of gp120. Intensive washing with 0.05% Tween phosphate-buffered saline (PBS-T) was carried out to remove any unbound phage. As the family-specific phage library was assumed to contain only phages that recognize the CD4bs, a general trypsin (1 mg/ml) elution was used to harvest the bound phages instead of a competitive sCD4 elution originally used to isolate the parental A12/D7 VHH. The trypsin was then neutralized with 210 μM trypsin blocker acetylbenzenesulfonyl fluoride (Sigma). Selections where a larger number of clones were eluted than blank controls, while keeping background to the minimum, were taken forward to a second round of panning. Selections were carried out for two rounds. Genes from selected VHH were recloned in an expression vector (pAX51), which lacks the phage-derived gene 3. The VHH is produced with a C-terminal hexahistidine tail under the control of the *lac* promoter (35). Following transformation into *E. coli* TG1 cells, individual colonies were picked and cultivated in 2× TY medium containing 100 μg/ml ampicillin and 0.1% glucose. Expression was induced by 0.5 mM isopropyl-β-D-galactopyranoside for 3 h as soluble periplasmic proteins. VHH chosen for further characterization were then purified by immobilized metal affinity chromatography using TALON metal affinity resin (Clontech) and then dialyzed against phosphate-buffered saline.
Library of Family-specific Llama Antibody Fragments

Competition ELISA—An ELISA was carried out to determine whether the selected VHH were able to compete with sCD4 for binding to gp120, and performed as described previously (30). Briefly, 10 μg/ml recombinant sCD4 containing the D1–D3 regions of CD4, obtained from the Centralized Facility for AIDS Reagents (National Institute for Biological Standards and Control, Potters Bar, UK), was coated overnight on 96-well Maxisorp plates. After blocking with 4% skim milk, 1 μg/ml IIIBgp120 was preincubated for 1 h with serial dilutions of VHH and negative control VHH and then subsequently added onto the plates for 1 h. The mAb b12, which targets the CD4bs of gp120, was included for comparison. Bound gp120 was detected with 10 ng/ml horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody (Dako, Denmark). SureBlue TMB microwell substrate (Kirkegaard & Perry Laboratories) was added and left to develop for 30 min, after which the reaction was stopped with 1 M HCl. The absorbance was measured at 450 nm.

Surface Plasmon Resonance—The kinetic binding parameters were determined with Biacore®. IIIBgp120 was diluted in 10 mM sodium acetate, pH 4.0, to a concentration of 15 μg/ml and injected over the surface at a flow rate of 5 μl/min for coupling via free amines to the CM5 sensor chip. Excess activated groups were blocked using a 7-min injection of 1 mM ethan-olamine, pH 8.5, at a flow rate of 5 μl/min. Purified VHH was diluted in HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% P20 surfactant) to 75, 50, 40, 30, and 20 nM, and the samples were injected for 2 min. A reference surface where no compound was immobilized was also included. Association was measured for 3 min and dissociation for 15 min. Regeneration was achieved by washing with 10 mM HCl for 3 min. The kinetic constants (i.e., the second-order rate constant for the association, kₐ, and the first-order rate constant for the dissociation, kₓ) were computed from the sensograms using the BIAEvaluation software (1:1 interaction), and the equilibrium dissociation constant (K_D) was calculated from kₐ/kₓ.

Mutation of VHH—Site-directed mutagenesis of the YYD₁₀² motif at the C terminus of the CDR3 was performed by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) in conjunction with the appropriate primers according to the manufacturer’s instructions. The mutations were verified by sequencing, followed by the expression and purification of VHH as described above, and are listed in Table 4. These were tested for binding in ELISA and for its neutralization potency in the TZM-bl assay.

Viruses—HIV-1 IIIB was obtained from the Centralized Facility for AIDS reagents (National Institute for Biological Standards and Control, Potters Bar, UK) and propagated in H9 cells. The subtype C Env gp120 clones, C2229 and 92BR025 (42), were introduced into the pHXB2Δenv vector (43) to produce replication-competent chimeric viruses after transfection in 293T cells. The QH0692.42, PVO.4, and ZM214M clones are part of the subtype B and C HIV-1 Reference Panels of Env

5 Koh, W. W. L., Forsman, A., Hué, S., van der Velden, G. J., Yirrell, D. L., McNight, A., Weiss, R. A., and Aasa-Chapman, M. M. I. (2010) J. Gen. Virol., in press.
containing inserts was determined to be 91% (data not shown). To our knowledge, this is the first published instance to describe the creation of a family-specific library containing homologous single-chain antibodies recognizing a specific epitope. Although the original A12 and D7 VHH were competitively eluted with soluble CD4 to obtain antibodies that can inhibit CD4 binding to gp120, a general elution method using trypsin was used here to elute all bound phages, as all the VHH in the family-specific library were assumed to be related to A12/D7. The creation of the A12/D7 family-specific VHH was therefore successful.

Variations in Neutralization Potencies—Thirty one different VHH from the family-specific library were brought forward for evaluation in HIV-1 neutralization assays. The VHH were selected based on differences in their amino acid sequences, especially in the CDR3 loop regions, and they included the 15 VHH tested for CD4 inhibition (see above). The parental A12 and D7 VHH competed with sCD4 for binding to gp120 in both the ELISA and the surface plasmon resonance assays (30), a competition ELISA was carried out to determine whether the newly selected cousins from the family-specific VHH isolation recognized a similar epitope on gp120. A fixed amount of IIIB gp120 was first allowed to bind to titrated amounts of VHH. The ability of the bound VHH to inhibit the interaction of gp120 with CD4 coated onto the solid phase was then measured.

Fifteen different VHH from the different outputs representing a range of different affinities were tested in this assay, together with an irrelevant VHH as negative control. The parental D7 VHH and a CD4bs human mAb, b12 (50), were also included in this assay. From Fig. 2, all the members of the family-specific VHH, together with the D7 VHH and mAb b12, are shown to compete with sCD4 for binding onto gp120 in a dose-dependent manner. In contrast, the irrelevant negative control VHH had no effect on sCD4 binding. Hence, although a specific elution strategy was not used in the biopanning, all of the selected VHH were found to recognize gp120 in a similar fashion to A12/D7. The creation of the A12/D7 family-specific VHH was therefore successful.

### Summary of outputs from selections

A summary of the selection conditions used in the biopanning process is shown. Two outputs of 94 clones each were collected, derived from selections with different antigen concentrations, giving a total of 376 clones.

| Outputs | 1st round | 2nd round |
|---------|-----------|-----------|
| 2B10    | 0.1 nM IIIB gp120 | 1 pM IIIB gp120 |
| 2B12    | 0.01 nM IIIB gp120 | 1 pM IIIB gp120 |
| 2C7     | 10 nM 92BR025 gp120 | 10 pM 92BR025 gp120 |
| 2C9     | 1 nM 92BR025 gp120 | 10 pM 92BR025 gp120 |

Selected VHH Inhibit Binding of sCD4 to gp120—As the parental A12 and D7 VHH competed with sCD4 for binding to gp120 in both the ELISA and the surface plasmon resonance assays (30), a competition ELISA was carried out to determine whether the newly selected cousins from the family-specific VHH isolation recognized a similar epitope on gp120. A fixed amount of IIIB gp120 was first allowed to bind to titrated amounts of VHH. The ability of the bound VHH to inhibit the interaction of gp120 with CD4 coated onto the solid phase was then measured.
which consisted of three subtype B viruses (IIIB, QHO692.42, and PVO.4) and three subtype C viruses (C222, 92BR025.C1, and ZM214M.PL15). Neutralization was assayed using the TZM-bl cell line. TZM-bl cells contain a Tat-dependent luciferase reporter system, which is only induced after HIV-1 infection. Neutralization by VHH was measured as the reduction of relative light units emitted compared with virus without VHH. TZM-bl cells express CD4 and the coreceptors, CXCR4 and CCR5, and thus they are sensitive to infection by all HIV-1 strains. The VHH concentration required to achieve 50% reduction of infectivity was determined, and the results are shown in Table 2. The parental A12 and D7 were included for comparison.

All VHH tested displayed similar neutralizing activity against the three subtype B viruses assayed; they were highly effective against IIIB (IC_{50} < 1 µg/ml), moderately potent against QHO692.42 (10 < IC_{50} < 25 µg/ml), and ineffective against PVO.4 (IC_{50} > 25 µg/ml). However, a different pattern emerged when the VHH were tested on the subtype C viruses. None of the VHH were able to neutralize ZM214M.PL15, but they showed marked differences in their ability to neutralize C222 and 92BR025.C1. Based on their IC_{50} values for C222 and 92BR025.C1, the VHH can be grouped into three categories named Broad, Intermediate, and Narrow. VHH in the Broad category were able to neutralize both C222 and 92BR025.C1 to high potency (IC_{50} < 1 and < 5 µg/ml, respectively), whereas VHH in the Intermediate category were able to neutralize C222 (0.5 < IC_{50} < 5.0 µg/ml) but not 92BR025.C1 (IC_{50} > 25 µg/ml).

Sequence Analysis of the CDRs—
All 31 VHH from the family-specific library possess unique sequences with variations seen within the framework regions as well as in the CDRs. To understand the molecular basis of the variation in neutralizing activity, we studied the amino acid sequences of the CDRs as they are crucial for antigen binding and specificity (51). The amino acid positions of the CDRs are defined using Chothia numbering as follows: 26–32 for CDR1, 52–56 for CDR2, and 95–102 for CDR3 (52) and are grouped according to their three neutralizing categories (Table 3).

Certain residues within the CDRs were found to show mutations in high frequencies. These are residues 28, 31, and 32 within CDR1, residue 56 in CDR2, and residues 100D, 101, and 102 in CDR3. These “hot spot” residues, except for 101 and 102 in CDR3, are also found in VHH with Broad neutralizing activity and are therefore unlikely to compromise the neutralization ability of the VHH on the viruses tested, but might affect the range of IC_{50} values observed for C222 and 92BR025.C1 within the Broad category.

When analyzing residues 101 and 102, a distinct YYD^{102} motif at the C-terminal end of the CDR3 loop is conserved within the Broad neutralizer category but absent from the Narrow neutralizer category. Mutation of this YYD^{102} motif to YYY, YNY, or YND seemed to hamper neutralizing potency against the subtype C viruses C222 and 92BR025.C1. For instance, five of the eight Narrow neutralizers (2B10E8, 2B10E2, 2C9F6, 2B10B10, and 2C7D1) and two of the three Intermediate neutralizers (2C7H5 and 2C9E2) have CDR sequences that are identical to those found in one or more Broad neutralizers, except for residues 101 (Tyr to Asn substitution) and/or 102 (Asp to Tyr substitution). Paired groups marked with an asterisk in Table 3 have a Y101N substitution, whereas VHH marked with a double-plus sign have Y101N and D102Y mutations, and those marked with a plus sign have Y101N and/or D102Y mutations.

To confirm that the YYD^{102} motif is crucial for the Broad neutralizing ability of the VHH, we extended the sequence analysis to include framework sequences and searched for VHH cousins with identical sequences but varying in the YYD^{102} motif at the C-terminal end of the CDR3 loop is conserved within the Broad neutralizer category but absent from the Narrow neutralizer category. Mutation of this YYD^{102} motif to YYY, YNY, or YND seemed to hamper neutralizing potency against the subtype C viruses C222 and 92BR025.C1. For instance, five of the eight Narrow neutralizers (2B10E8, 2B10E2, 2C9F6, 2B10B10, and 2C7D1) and two of the three Intermediate neutralizers (2C7H5 and 2C9E2) have CDR sequences that are identical to those found in one or more Broad neutralizers, except for residues 101 (Tyr to Asn substitution) and/or 102 (Asp to Tyr substitution). Paired groups marked with an asterisk in Table 3 have a Y101N substitution, whereas VHH marked with a double-plus sign have Y101N and D102Y mutations, and those marked with a plus sign have Y101N and/or D102Y mutations.
motif. In Fig. 3A, the sequences of 2C7D2 (a Broadly neutralizing VHH) and 2C9E2 (an Intermediate neutralizing VHH) are identical except for a single point mutation (D102Y) to produce a YYY102 motif. Likewise, in Fig. 3B, a double mutation from YYD102 to YNY102 is the only difference between the two VHH 2B1G5 and 2C7D1, which display a Broad and Narrow phenotype, respectively. VHH with a single Y101N mutation to produce the YND102 motif can display either an Intermediate or Narrow phenotype, as demonstrated by 2C7H5 and 2C7E11 in Fig. 3C. These two VHH contain a few other mutations in their sequences, but because those residues are also found in other VHH within the Broad category, they are unlikely to have an impact on neutralizing activity. The YYY102, YNY102, and YND102 motifs are therefore associated with a less broad neutralizing potential, and many more VHH variants within the family-specific library exist to support this conclusion.

Interestingly, one variant VHH 2C7F7 possessed the YYD102 motif but fell under the Intermediate category. This VHH (Fig. 3D) contains three unique mutations (H32F, M34L, and A40G) that are not found in any other VHH within the family and involve changes in the hydrophobicity of the residues. For example, a unique phenylalanine residue that is nonpolar and very hydrophobic had replaced the polar histidine or tyrosine residues that are usually found in position 32 within CDR1. Together, these cumulative mutations may have an effect on antigen binding and thus compromise the potential for the Broad phenotype conferred by the YYD102 motif.

Mutations in the YYD102 Motif—To confirm the significance of the YYD102 motif at the end of the CDR3 and its associated effect on the recognition and neutralization of HIV-1, eight different mutations were generated in the YYD102 and YNY102 motif of the parental A12 and D7, respectively. The residues in positions 101 and 102 were mutated to determine the contribution of each residue, and a list of the mutants is shown in Table 3. Mutations in either or both these residues did not abrogate binding to IIIBgp120 in ELISA, and all mutants were still able to neutralize HIV-1 IIIB to high potency (IC50 <1 μg/ml).

Mutants 1–3 are point mutations of A12 where Tyr101 was changed into Ala101, Asn101, and Gln101, respectively. When compared with A12, mutants 1 and 2 lost the ability to neutralize both C222 and 92BR025.C1 with just a single amino acid change. Mutant 3 lost the ability to neutralize 92BR025.C1 and a 200-fold drop in potency against C222. Mutants 4 and 5 are point mutants of A12 where Asp102 was changed into Ala102, Asn102, and Gln102, respectively. When compared with A12, there was no significant loss in neutralizing activity.

Mutants 6–8 are point mutations of D7 where Tyr101 was changed into Ala101, Asn101, and Gln101, respectively. When compared with D7, there was no significant loss in neutralizing activity.

Mutants 9–12 are point mutations of D7 where Asp102 was changed into Ala102, Asn102, and Gln102, respectively. When compared with D7, there was no significant loss in neutralizing activity.
TABLE 5
Binding affinities of VHH

| VHH       | $k_a$ | $k_d$ | $K_D$ | Motif Neutralization category |
|-----------|-------|-------|-------|------------------------------|
| A12       | 2.73  | 0.298 | 1.00  | YYD Broad                     |
| 2B10H1    | 1.48  | 0.252 | 0.77  | YYD Broad                     |
| 2B10H3    | 1.64  | 0.312 | 0.52  | YYD Broad                     |
| 2BG6      | 1.47  | 0.309 | 0.21  | YYD Broad                     |
| 2B10D1    | 1.12  | 0.247 | 0.22  | YYD Broad                     |
| 2B10C2    | 1.20  | 0.276 | 0.23  | YYD Broad                     |
| 2B10A2    | nd    | 0.680 | nd    | YYD Broad                     |
| 2B10B4    | 1.65  | 0.856 | 0.52  | YYD Broad                     |
| 2B10D7    | 1.29  | 0.737 | 0.57  | YYD Broad                     |
| 2B10D5    | 1.15  | 0.761 | 0.66  | YYD Broad                     |
| 2B10E8    | 1.02  | 1.12  | 1.1   | YND Narrow                    |
| 2B10B10   | 1.99  | 3.39  | 1.7   | YNN Narrow                    |
| 2B10E2    | 1.67  | 3.18  | 1.9   | YNY Narrow                    |
| D7        | 1.55  | 5.51  | 2.9   | YNY Narrow                    |

FIGURE 3. Sequence comparison of VHH pairs. Alignments of paired amino acid sequences of closely related VHH that display different neutralization phenotypes were compared. Identical residues are denoted in dashes, and the neutralization category of each VHH is denoted in square brackets (B, Broad; I, Intermediate; N, Narrow). A, YYD to YYN mutation resulted in a change from Broad to Intermediate category. B, double mutation from YYD to YYN resulted in a change from Broad to Narrow category. C, switch from YYD to YYN can result in a change from Broad to Intermediate and Narrow category. D, mutations in the other VHH in the Broad category. D, despite having a YYD motif in 2C7F7, a YYD to YYY mutation resulted in a change from Broad to Narrow category. The H32F mutation is within the CDR1.

TABLE 4
Characterization of A12/D7 mutants

| Mutant No. | Parent | Motif – 101-102 | ELISA | Neutralization IC50 (μg/ml) | Category |
|------------|--------|-----------------|-------|----------------------------|----------|
| A12        | -      | YYD             | Yes   | 0.01                       | Broad    |
| 1          | A12    | YYD             | Yes   | 0.2                        | Narrow   |
| 2          | A12    | YND             | Yes   | 0.12                       | Narrow   |
| 3          | A12    | YYD             | Yes   | 0.08                       | Intermediate |
| 4          | A12    | YNY             | Yes   | 0.04                       | Intermediate |
| 5          | A12    | YYE             | Yes   | 0.02                       | Narrow   |
| 6          | A12    | YNY (D7-like)   | Yes   | 0.88                       | Narrow   |
| 7          | D7     | YND             | Yes   | 0.11                       | Narrow   |
| 8          | D7     | YYD (A12-like)  | Yes   | 0.04                       | Broad    |
| A12        | -      | YYN             | Yes   | 0.07                       | Narrow   |

In this study, we have successfully used a novel method to create a family-specific library of HIV-1-neutralizing VHH-containing variants that are homologous to the parental A12/D7 VHH that neutralize a broad spectrum of HIV-1 isolates (30). This was achieved through the use of specially designed primers that target the tail of the CDR3 sequence, thereby isolating sequence-homologous VHH from the diverse repertoire that have affinity matured in the immunized llama. The CDR3 was targeted in this study as its importance in the classification of antibodies with similar epitope recognition was described for HIV-specific antibodies from human donors (53) and for VHH targeting bacteriophage proteins (41). The family-specific library was then panned on picomolar concentrations of recombinant gp120 in solution form to reduce potential avidity effects. As all the variants within the family were assumed to target the CD4bs of gp120 in a similar fashion to A12/D7, a general trypsin elution was employed to elute all bound phages. This method could elute out phages that might not be eluted by soluble CD4 elution. This property of the family-specific library was confirmed with a competition ELISA experiment where all the isolated VHH tested were able to pre-
vent soluble CD4 from binding to gp120. It is possible that post-translational modifications of VHH when expressed in mammalian cells might affect their properties, but this has not been studied to date. A high resolution crystal structure study of A12 bound to gp120 is currently being sought, which would help to elucidate the precise binding epitope of this VHH.

Through this novel technique, we have isolated 49 unique VHH belonging to the A12/D7 family that had not been previously isolated when using the competitive elution method with soluble CD4. These variants were able to discriminate between two of the subtype C viruses tested, and these VHH were grouped according to their ability to neutralize the virus isolates tested. The molecular basis of this discrimination was mapped to the last two amino acid positions at the C terminus of the CDR3 loop. The YYD102 motif is correlated with the ability to neutralize both subtype C HIV-1 Envs. Instead of the painstaking task of antibody engineering, the mining of a diverse pool of antibodies that has been studied to date. A high resolution crystal structure study of the D7 VHH has recently been solved and will help to shed some light on structure and function (54). There is also a direct correlation between the YYD102 motif and high binding affinities to IIIB gp120, with changes within the motif resulting in decreased affinities. Antibody affinities in the high picomolar range are close to the suggested affinity ceiling for in vivo maturated antibodies (55, 56), which might explain the absence of VHH with even higher affinities than A12 in the original immune library.

The diversity of HIV-1 viral envelopes restricts the generation of potent and broadly cross-reactive Nabs (57) and is a major obstacle for effective antibody-based immunization against HIV-1. The creation of such new antibodies is a high priority for HIV-1 vaccine development. This method can help to create a diverse panel of neutralizing antibodies that has the ability to recognize different virus isolates. The panel of VHH can be used for identification and characterization of conserved epitopes on HIV-1 envelope proteins that can serve as templates for the design of new immunogens, and also for potential use as therapeutics.

In conclusion, we have created a family-specific VHH library whereby members of the family show similarity in its ability to recognize a particular antigen. However, the diverse members of the family show variations in terms of their binding affinities, as well as neutralization ability across different strains of viruses. By analyzing the sequences of the VHH, we were able to associate certain amino acid residues as crucial to potent neutralization capacity and better recognition of a diverse set of HIV-1 Envs. Instead of the painstaking task of antibody engineering, the mining of a diverse pool of antibodies that has undergone in vivo affinity maturation has led to the creation of a panel of homologous antibodies, allowing the study of the paratope that is essential for Env recognition and neutralization. These VHH may provide a basis for future engineering of antibodies against HIV-1 and also offer new tools for development of inhibitory microbicides, vaccines, and research reagents.

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Library of Family-specific Llama Antibody Fragments