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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 IIIB 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-

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[4] 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.

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 (vTF7-3, 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 YM-50 centrifugal filter units (Millipore) with a 50-kDa cutoff, and the integrity of the biotinylated proteins was verified in 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 transformed 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 neutravidin (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 in 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 1-thio-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.

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 μg/ml of the sheep polyclonal antibody D7324 (Aalto BioReagents) and then with 0.5 μg/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 M ethanolamine, 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, k_a, and the first-order rate constant for the dissociation, k_d) were computed from the sensograms using the BIAEvaluation software (1:1 interaction), and the equilibrium dissociation constant (K_D) was calculated from k_d/k_a.

Mutation of VHH—Site-directed mutagenesis of the YYD^{102} 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, C222[5] 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 Clones (44, 45) and were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. These HIV-1 envelope pseudotyped viruses were produced in 293T cells by cotransfection with the pSG3Δenv plasmid.

HIV-1 Neutralization Assays—The neutralizing ability of the VHH was tested against a panel of HIV-1 viruses using a luciferase-based assay in TZM-bl cells (46–48), which was obtained through the AIDS Research and Reference Reagent Program, National Institutes of Health, from J. C. Kappes, X. Wu, and Tranzyme, Inc. This assay measures the reduction in levels of Tat-driven luciferase reporter gene expression following a single round of virus infection in TZM-bl cells. Briefly, 3-fold serial dilutions of purified VHH starting from 25 μg/ml were performed in duplicate in 10% (v/v) fetal calf serum-supplemented Dulbecco's modified Eagle's growth medium (Invitrogen) using 96-well flat-bottom culture plates (Nuclon, Nunc). 200 $TCID_{50}$ titers of virus were then added to each well, and the plates were incubated for 1 h at 37 °C. TZM-bl cells were subsequently added (1×10^4 cells/well) in growth medium supplemented with DEAE-dextran (Sigma) at a final concentration of 11 μg/ml. Assay controls included replicate wells of TZM-bl cells alone (background control) and TZM-bl cells with virus assayed (virus control). No virus inactivation was observed with an irrelevant negative control VHH. Following a 48-h incubation at 37 °C, all but 100 μl of the assay medium was removed, and 100 μl of Bright-Glo luciferase reagent (Promega, Madison, WI) was added to each well. The cells were allowed to lyse for 2 min, and the luminescence was measured using a luminometer. The 50% inhibitory concentration (IC_{50}) titers were calculated as the VHH concentration that achieved a 50% reduction in relative luminescence units compared with the virus control relative luminescence units, after subtraction of the background control relative luminescence units from both values. The calculations were performed using the XLFit4 software (ID Business Solutions, Guildford, UK), and the assay was performed on at least two separate occasions.

RESULTS

Construction of the A12/D7 Family-specific Phage Display Library—Both the A12 and D7 VHH were isolated from a llama that was immunized with recombinant gp120 derived from HIV-1 CN54 (subtype B′/C, CRF07_BC) and were able to cross-neutralize various subtypes and strains of HIV-1 (30). Because the CDR3 possesses the greatest flexibility and conformational variability of the CDRs, it could have the greatest influence on antigen binding (49). As A12 and D7 share similar sequences, we designed an A12/D7-specific degenerate primer that recognized the C-terminal stretch of nucleotides in the CDR3 loop and the first four (conserved) amino acids of framework 4 of A12/D7 to amplify all the affinity-maturated antibodies that share sequences homologous to the A12 and D7 VHH. RNA extracted from the peripheral blood lymphocyte pool of the original immunized llama was amplified by PCR with the A12/D7-specific primer in conjunction with a primer to a highly conserved framework 1 region, and an A12/D7 family-specific phage display library was generated (Fig. 1). The size of this library was estimated to be 2.0×10^6 clones, and the proportion of clones

[5] Koh, W. W. L., Forsman, A., Hué, S., van der Velden, G. J., Yirrell, D. L., McKnight, Á., Weiss, R. A., and Aasa-Chapman, M. M. I. (2010) *J. Gen. Virol.*, in press.

(A)

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 FR1 primer →
A12          GCGGTGCAGCTGGTGGAGTCTGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAGACTC 60
D7           GCGGTGCAGCTGGTGGAGTCTGGGGGAGGATTGGCGCAGGCTGGGGGCTCTCTGAGACTC 60

A12          TCCTGTACAGCCTCTGGACGCATCAGCAGTAGCTATGATATGGGCTGGTTCCGCCAGGCT 120
D7           TCCTGTACAGTTTCTGGACGCACCAGTAGTAGCCATGATATGGGCTGGTTCCGCCAGGCT 120

A12          CCAGGGAAGGAGCGTGAGTTTGTAGCGGCTATTAGTTGGAGTGGTGGTACCACAGACTAT 180
D7           CCAGGAAAGGAGCGTGAGTTTGTAGCGGCTATTAGCTGGAGTGGTGGTACCACAAACTAT 180

A12          GCAGACTCCGTGAAGGGGCGATTCGCCATCTCCAAAGACAACGCCAAGAACGCAGTGTCC 240
D7           GCAGACTCCGTGAAGGGGCGATTCGCCATCTCCAAAGACAACGCCAAGAACGCAGTCTCC 240

A12          CTGCAAATGAACAGCCTGAAACCCGAGGACACGGCCGTTTATTACTGTGCAGCTAAGTGG 300
D7           CTGCAAATGAACAGCCTGAAACCTGAGGACACGGCCGTTTATTACTGTGCAGCTAAGTGG 300

A12          CGACCGCTACGTTATAGTGACTACCCTTCGAATTCGGATTACTACGACTGGGGCCAGGGG 360
D7           CGACCGCTACGTTATAGTGATAACCCTTCGAATTCAGATTATAACTACTGGGGCCAGGGG 360
                                        ← A12/D7 primer

A12          ACCCAGGTCACCGTCTCCTCA 381
D7           ACCCAGGTCACCGTCTCCTCA 381
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(B)

A12/D7-specific primer:

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5'- TGAGGAGACGGTGACCTGGGTCCCCTGGCCCCAGTMGTWRTAATCYGAATTCG -3'
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FIGURE 1. **Nucleotide sequences of A12 and D7 (*A*) and the A12/D7-specific primer (*B*) are shown with the CDR3 loop-encoding region shaded in *gray*.** A unique degenerate reverse primer was designed (*B*) to extend into the CDR3 region and is complementary to the *underlined* sequence in *A*.

TABLE 1

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

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.

Selection and Screening of VHH—Biopanning was carried out on two different antigens, IIIB gp120 (subtype B) and 92BR025 gp120 (subtype C), in parallel. As the A12/D7 VHH was originally panned on IIIB gp120, adding a subtype C Env may promote the selection of VHH with cross-subtype recognition properties. The recombinant 92BR025 gp120 was cloned directly from a primary isolate and expressed in a mammalian expression system. To remove avidity effects, the phages were allowed to bind to biotinylated gp120 in solution, before capturing the bound phages on either neutravidin or the anti-gp120 Ab D7324-coated plates.

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 and therefore specific for the CD4bs on gp120. Two rounds of selections were carried out on both antigens in parallel, and the outputs that gave the best enrichment of binders from the optimum concentration of antigen were brought forward to be subcloned into an expression vector providing a C-terminal His_6 tag for purification. Ninety four clones were picked from each output, a summary of which is shown in Table 1. A restriction analysis of the selected clones showed low variations in diversity, which was expected as they were all part of the same family (data not shown). The clones were further characterized by sequencing.

Out of the total 376 clones picked, there were 49 unique amino acid sequences. Although two different antigens were used in the biopanning, identical clones were found in both selection strategies. All newly isolated VHH showed high sequence homology to the parental A12/D7 VHH, but variations in the sequences were observed in the framework regions, as well as the complementary-determining regions of the VHH.

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.

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.

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 had been tested in the TZM-bl assay against a broad panel of T-cell line adapted viruses, cloned Env pseudotyped viruses, as well as cloned Env replication-competent chimeric viruses, and A12 was also shown to have neutralizing activity in a peripheral blood mononuclear cell assay (30). To study the 31 VHH in detail, the number of viruses tested was narrowed down to a smaller panel of six viruses,

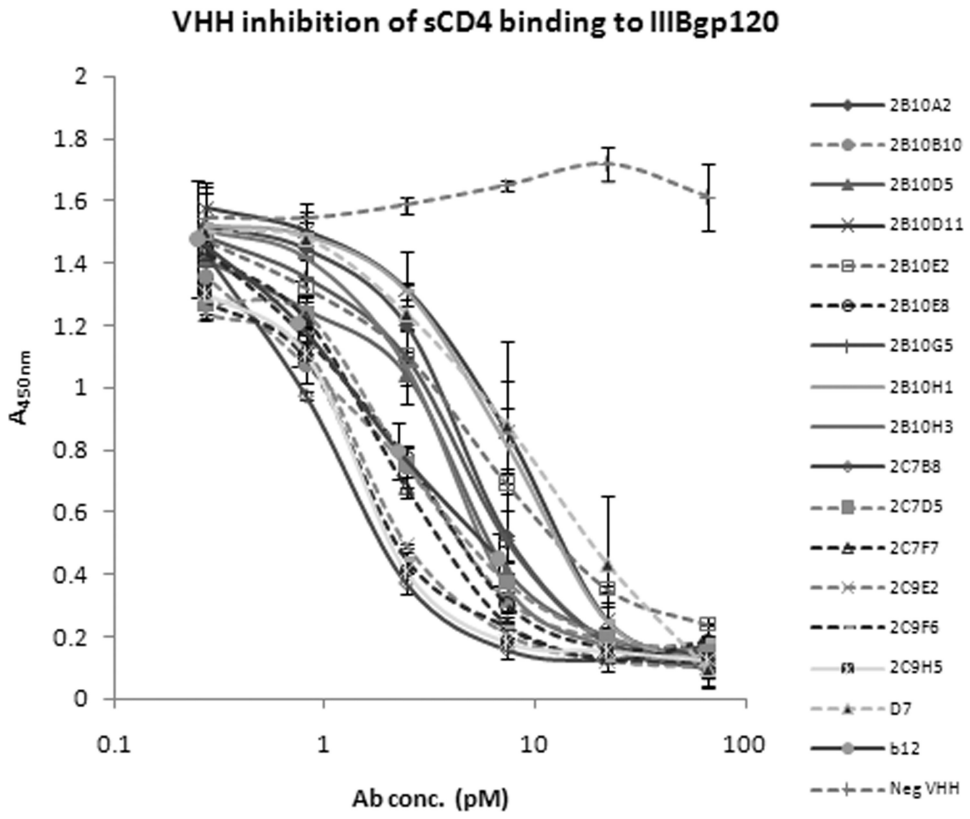

FIGURE 2. **VHH inhibition of sCD4 binding to gp120.** A selection of 15 VHH from the A12/D7 family-specific library was tested for their ability to inhibit sCD4 binding to gp120, together with the parental D7 VHH and the mAb b12. All of them were found to inhibit sCD4 binding in a dose-dependent manner. An irrelevant VHH was used as a negative control, and this did not affect sCD4 binding. *Ab conc.*, antibody concentration.

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). Finally, VHH in the Narrow category were unable to neutralize both C222 and 92BR025.C1 (IC_{50} >25 μg/ml). Within the Broad category, the ranges of IC_{50} values for C222 and 92BR025.C1 were ~10- and 2-fold, respectively. The parental A12 falls into the Broad category, whereas D7 falls into the Narrow category.

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}

TABLE 2

VHH IC_{50} (μg/ml) titers against HIV-1

VHH from the family-specific library, together with the parental A12 and D7, were tested for their potency in neutralization against the indicated viruses as described in the text. The VHH were categorized into Broad, Intermediate, or Narrow, according to their potency against C222 and 92BR025.C1. To aid comprehension, the titers have been shaded, with darker colors indicating more potent neutralization. nd indicates not determined.

	VHH	VHH Neutralization shown as IC_{50} (μg/ml)					
		Subtype B			Subtype C		
		IIIB	QH0692.42	PVO.4	C222	92BR025.C1	ZM214M.PL15
Broad	2B10A2	0.07	19.73	>25	0.13	2.01	>25
	2B10C2	0.16	18.66	>25	0.12	1.57	>25
	2B10D5	0.57	19.63	>25	0.96	1.95	>25
	2B10D7	0.12	21.63	>25	0.20	1.42	>25
	2B10D11	0.18	15.48	>25	0.36	4.47	>25
	2B10G5	0.40	16.10	>25	0.04	0.28	>25
	2B10H1	0.29	17.21	>25	0.08	0.74	>25
	2B10H3	0.29	19.14	>25	0.06	0.39	>25
	2C7B8	0.15	10.78	>25	0.27	0.78	>25
	2C7D2	0.17	20.71	>25	0.28	0.60	>25
	2C7E3	0.07	12.16	>25	0.13	0.48	>25
	2C7G11	0.16	16.08	>25	0.24	1.76	>25
	2C9A8	0.30	nd	>25	0.41	2.40	>25
	2C9B11	0.24	nd	>25	0.30	3.41	>25
	2C9C5	0.12	nd	>25	0.16	1.23	>25
	2C9D9	0.37	nd	>25	0.61	3.64	>25
	2C9E4	0.35	nd	>25	0.50	1.76	>25
	2C9E7	0.30	20.87	>25	0.34	0.90	>25
	2C9F1	0.13	19.31	>25	0.48	0.95	>25
	2C9F10	0.13	18.70	>25	0.16	0.61	>25
	2C9H5	0.17	17.28	>25	0.19	0.73	>25
Inter-mediate	2C7F7	0.34	17.31	>25	4.53	>25	>25
	2C7H5	0.12	16.50	>25	10.20	>25	>25
	2C9E2	0.20	19.39	>25	4.08	>25	>25
Narrow	2B10B10	0.54	19.40	>25	>25	>25	>25
	2B10E2	0.46	15.90	>25	>25	>25	>25
	2B10E8	0.49	18.54	>25	>25	>25	>25
	2C7D1	0.24	16.29	>25	>25	>25	>25
	2C7D5	0.13	20.04	>25	>25	>25	>25
	2C7E11	0.21	17.27	>25	>25	>25	>25
	2C9F6	0.18	14.54	>25	>25	>25	>25
	A12	0.09	13.00	>25	0.07	0.23	>25
	D7	0.30	17.00	>25	>25	>25	>25

TABLE 3

Sequence comparison of the CDRs

The VHH are grouped into the three different neutralization categories, and the amino acid sequences of their CDRs are aligned and compared. The consensus sequence with Chothia numbering to denote the CDR position (52) is shown in the top row. Residues that are identical to the consensus sequence are shown in dashes. Variants of the YYD^{102} motif at the end of the CDR3 are shown in boldface type, and the YYD^{102} motif is underlined. The symbols *, †, and ‡ are used to denote groups of VHH with similar CDR sequences but with variations only in the YYD^{102} motif. (*, Y101N substitution; ‡, Y101N and D102Y mutations; †, Y101N and/or D102Y mutations.)

VHH	CDR1	CDR2	CDR3
	26 32	*52ABC3456*	*95 100ABCDEFGHIJ 102*
Consensus	GRISSSH	SWSGGTTD	KWRPLRYSDNPSNSD**YYD**
Broad Neutralizers			
2B10D5*	--T----	--------	----------
2B10C2*	--T----	--------	----------
2C9A8	--T----	--------	-------N----------
2C9B11	--T----	--------	-------N----------
2B10A2	--T----	-------N	----------
2B10D11	-------	--------	----------
2C7E3	-------	-------N	---------S----------
2C9C5	-------	-------N	---------S----------
2C9F10	-------	-------N	---------S----------
2C7D2†	-------	--------	---------D----------
2B10G5†	-------	--------	---------D----------
2C9E7†	-------	--------	---------D----------
2B10H3	-------	----A---	---------D----------
2C7B8	------Y	-----A--	---------Y----------
2B10D7‡	------Y	--------	---------Y----------
2B10H1‡	------Y	--------	---------Y----------
2C9F1	------Y	------K-	---------Y----------
2C9H5	------Y	---S----	---------Y----------
2C7G11	------Y	--------	---------S----------
2C9D9	-----MY	-------A	---------D----------
2C9E4	-----MY	-------A	---------D----------
A12	------Y	--------	---------Y----------
Intermediate Neutralizers			
2C7H5†	-------	--------	---------D------**N-**
2C9E2†	-------	--------	---------D-------**Y**
2C7F7	--T---F	--------	----------
Narrow Neutralizers			
2B10E8*	--T----	--------	----------**N-**
2C7E11	--V----	--------	--------E-------**N-**
2B10E2‡	------Y	--------	---------Y------**NY**
2C9F6	------Y	--------	---------Y-------**Y**
2B10B10‡	------Y	--------	---------Y------**NY**
2C7D1	-------	--------	---------D------**NY**
2C7D5	--L--MY	-----S-T	----------**N-**
D7	--T----	-------N	----------**NY**

motif. In Fig. 3*A*, 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 $\mathbf{YYY}^{102}$ motif. Likewise, in Fig. 3*B*, a double mutation from YYD^{102} to $\mathbf{YNY}^{102}$ is the only difference between the two VHH 2B10G5 and 2C7D1, which display a Broad and Narrow phenotype, respectively. VHH with a single Y101N mutation to produce the $\mathbf{YND}^{102}$ motif can display either an Intermediate or Narrow phenotype, as demonstrated by 2C7H5 and 2C7E11 in Fig. 3*C*. 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 YYY^{102}, YNY^{102}, and YND^{102} 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 YYD^{102} motif but fell under the Intermediate category. This VHH (Fig. 3*D*) 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 YYD^{102} motif.

Mutations in the YYD^{102} Motif—To confirm the significance of the YYD^{102} 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 YYD^{102} and YNY^{102} 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 4. 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 (IC_{50} <1 μg/ml).

Mutants 1–3 are point mutations of A12 where Tyr^{101} was changed into Ala^{101}, Asn^{101}, and Gln^{101}, 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 Asp^{102} was changed into Ala^{102}

```
(A)
2C7D2 [B]    EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY
2C9E2 [I]    ------------------------------------------------------------

2C7D2        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYDWGQ
2C9E2        ------------------------------------------------------Y---

(B)
2B10G5 [B]   EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY
2C7D1  [N]   ------------------------------------------------------------

2B10G5       ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYDWGQ
2C7D1        ------------------------------------------------------NY---

(C)
2B10G5 [B]   EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY
2C7H5  [I]   ------------------------------------------------------------
2C7E11 [N]   ---------------------------V--------------------------------

2B10G5       ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYDWGQ
2C7H5        ---------------S----------------------------------ND---
2C7E11       ------------------------------------------------EN--ND---

(D)
2B10C2 [B]   EVQLVESGGGLVQTGGSLRLSCTVSGRTSSSHDMGWFRQAPGKEREFVAAISWSGGTTDY
2C7F7  [I]   ------------------------------F-L-----G--------------------

2B10C2       ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDNPSNSDYYDWGQ
2C7F7        ------------------------------------------------------------
```

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 YYY mutation resulted in a change from Broad to Intermediate category. *B*, double mutation from YYD to YNY resulted in a change from Broad to Narrow category. *C*, switch from YYD to YND can result in a change from Broad to Intermediate and Narrow category. Mutations in the other residues are unlikely to significantly influence the neutralization phenotype as these amino acid substitutions are present in other VHH in the Broad category. *D*, despite having a YYD motif in 2C7F7, a unique three-point mutation resulted in a change from Broad to Intermediate category. The H32F mutation is within the CDR1.

TABLE 4

Characterization of A12/D7 mutants

Mutations in residues 101 and 102 were carried out on the parental A12 and D7 VHH. All the mutants were able to bind to IIIB gp120 in ELISA and neutralized HIV-1 IIIB with high potency. Mutants 1 and 2 lost the ability to neutralize both C222 and 92BR025 with just a point mutation change in residue 101. Mutant 5 retained the ability to neutralize both C222 and 92BR025 despite a D102E mutation. Mutants 6 and 8 are double back mutations where the YYD^{102} and YNY^{102} motifs were inter-switched, which resulted in a corresponding change in the neutralization phenotype. Residues 101 and 102 at the end of the CDR3 loop are therefore important for the subtype C Env specificity tested here.

Mutant No.	Parent	Motif – 100J-102	ELISA	Neutralization IC_{50} (μg/ml)			Category
				IIIB	C222	92BR025	
A12	-	YYD	Yes	0.01	0.03	0.25	Broad
1	A12	YAD	Yes	0.2	>25	>25	Narrow
2	A12	YND	Yes	0.12	>25	>25	Narrow
3	A12	YQD	Yes	0.08	6.63	>25	Intermediate
4	A12	YYA	Yes	0.04	0.97	>25	Intermediate
5	A12	YYE	Yes	0.02	0.09	0.71	Broad
6	A12	YNY (D7-like)	Yes	0.88	>25	>25	Narrow
7	D7	YND	Yes	0.11	>25	>25	Narrow
8	D7	YYD (A12-like)	Yes	0.04	0.05	1.27	Broad
D7	-	YNY	Yes	0.07	>25	>25	Narrow

and Glu^{102}, respectively. Mutant 5 retained the ability to neutralize both viruses, and mutant 4 lost the ability to neutralize 92BR025.C1 only. Therefore, a single point mutation in either residue 101 or 102 was sufficient to cause a phenotype change. It would also appear that residue 101 is more important in discriminating between the two viruses than residue 102. Mutants 6 and 8 contain two double back mutations where the YYD^{102} and YNY^{102} motifs of both A12 and D7 were inter-switched. These resulted in a corresponding neutralization phenotype change from Broad to Narrow and vice versa, thus confirming that the final two residues of the CDR3 loop are important in the neutralization of the subtype C viruses tested here.

TABLE 5

Binding affinities of VHH

The binding kinetics were determined for a sampling of VHH from the family-specific library, and the VHH were ranked according to decreasing affinity (K_D). The parental A12 and D7 took the pole ends of the table. VHH with a YYD^{102} motif have affinities of less than 1 nM, whereas VHH without a YYD^{102} motif have affinities of more than 1 nM, which is associated with a change in neutralization category from Broad to Narrow.

VHH	k_a	k_d	K_D	Motif	Neutralization category
	$10^5\ M^{-1}\ s^{-1}$	$10^{-4}\ s^{-1}$	nM		
A12	2.73	0.298	0.10	YYD	Broad
2B10H1	1.48	0.252	0.17	YYD	Broad
2B10H3	1.64	0.312	0.19	YYD	Broad
2B10G5	1.47	0.309	0.21	YYD	Broad
2B10D11	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	YNY	Narrow
2B10E2	1.67	3.18	1.9	YNY	Narrow
D7	1.55	5.51	2.9	YNY	Narrow

VHH Outputs Show Diverse Affinities—A selection of VHH from the family-specific library was tested in surface plasmon resonance to determine their affinity constants (K_D) with IIIBgp120. The affinities of the VHH ranged from 0.1 to 2.9 nM, representing a 30-fold difference in affinities. When the VHH were ranked according to their affinities as shown in Table 5, VHH with higher affinities of <1 nM possessed a YYD^{102} motif and invariably belong to the Broad neutralizer category, whereas VHH with lower affinities of >1 nM are without the YYD^{102} motif and belong to the Narrow neutralizer category. Therefore, there is a clear association with the YYD^{102} motif for higher affinities to IIIBgp120.

DISCUSSION

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 YYD^{102} motif is correlated with the ability to neutralize both subtype C HIV-1 envelopes, C222 and 92BR025.C1. Any change in this motif was sufficient to abrogate the ability to neutralize either or both viruses, and this was confirmed with mutational studies. Mutations in the other hot spot residues within the framework regions or CDRs did not affect this neutralization phenotype, although it might contribute to the wide range of IC_{50} values observed with C222 and 92BR025.C1. This would require further analysis to determine the contributory role of each residue involved in antigen specificity and binding to the various HIV-1 envelopes. A high resolution crystal structure 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 YYD^{102} 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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