Simian Immunodeficiency Virus (SIV) Envelope-Specific Fabs with High-Level Homologous Neutralizing Activity: Recovery from a Long-Term-Nonprogressor SIV-Infected Macaque

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An antibody phage display library was constructed from RNA extracted from lymph node cells of a simian immunodeficiency virus (SIV)-infected long-term-nonprogressor macaque. Seven gp120-reactive Fabs were obtained by selection of the library against SIV monomeric gp120. Although each of the Fabs was unique in sequence, there were two distinct groups based on epitope recognition, neutralizing activity in vitro, and molecular analysis. Group 1 Fabs did not neutralize SIV and bound to a linear epitope in the V3 loop of the SIV envelope. In contrast, two of the group 2 Fabs neutralized homologous, neutralization-sensitive SIVsm isolates with high efficiency but failed to neutralize heterologous SIVmac isolates. Based on competition enzyme-linked immunosorbent assays with mouse monoclonal antibodies of known specificity, these Fabs reacted with a conformational epitope that includes domains V3 and V4 of the SIV envelope. These neutralizing and nonneutralizing Fabs provide valuable standardized and renewable reagents for studying the role of antibody in preventing or modifying SIV infection in vivo.

Simian immunodeficiency virus (SIV) infection of macaques is a relevant and widely used animal model for human immunodeficiency virus type 1 (HIV-1) infection. SIV-infected macaques develop an AIDS-like syndrome characterized by declining peripheral $CD4^+$ cell counts, opportunistic infections, and wasting (11). As also observed in HIV-1-infected individuals, the duration of clinical latency in SIV-infected macaques varies widely, dependent upon the virulence of the infecting virus, as well as undefined host factors, which include the effectiveness of the immune response. The importance of host factors is underscored by the observation that animals experimentally infected with molecularly cloned SIV also exhibit considerable variation in disease outcome (15). While the majority of animals infected with pathogenic SIV isolates develop AIDS by 1 to 2 years postinfection, a small fraction of infected animals remain clinically healthy and appear to be the SIV macaque equivalent of human HIV-1 infected long-term nonprogressors (LTNP) (6, 35, 43). Although a vigorous humoral and cellular immune response accompanies both progressive and nonprogressive SIV infections, the mechanisms by which host immunity actually controls or protects against infection is not known. A role for neutralizing antibodies is suggested by the rapid death of animals that do not mount a SIV-specific antibody response (15, 53). However, the temporal association of measurable effector cytolytic CD8 suppression with down modulation of viral replication in the postacute phase of infection is also consistent with a role of cell-mediated immunity in controlling infection (27, 52).

The role of neutralizing antibody can be directly assessed through passive immunoprophylaxis experiments utilizing the SIV-infected macaque model. However, in considering SIV-infected macaques as a model to study the role of neutralizing antibody, one must take into consideration the fact that the immunodominant epitopes of the SIV and HIV envelope glycoproteins may differ. For example, although the V3 loop is an immunodominant region of both the HIV-1 and SIV envelope glycoproteins (31, 44), there are major differences in the characters of the V3 immune responses to these two viruses. This region of the HIV-1 envelope is antigenically variable and appears to represent a linear, type-specific, principal neutralizing domain of T-cell line-adapted isolates (18, 36) but not of primary isolates (48). Like primary HIV-1 isolates, the V3 analog of the SIV envelope is generally highly conserved and does not appear to constitute a neutralization epitope for SIV. Conformational epitopes may be the target of more broadly neutralizing antibody responses. Neutralizing antibodies to conformational epitopes are prevalent in sera from HIV-1-infected individuals (34, 45) and probably also in sera of SIV-infected macaques (19). Thus, 90% of the neutralizing activity in the serum of SIV-infected macaques is absorbed by a 45-kDa fragment of gp120 that encompasses domains V3 to V5 (20). In terms of the specific conformational epitopes of the two viruses, the majority of neutralizing monoclonal antibodies generated to HIV-1 appear to bind a conformational epitope involving the CD4 binding domain, whereas, this region has not been identified as a neutralizing domain of SIV. However, the repertoire of monoclonal antibodies generated to the SIV envelope is considerably less extensive than that of monoclonal antibodies generated to the HIV-1 envelope. Two neutralizing epitopes on SIV gp120 have been defined by mouse monoclonal antibodies, a linear V2 epitope and a conformational epitope that includes residues in the V3 and V4 domains (3, 22, 23, 25) but appears to be distinct from the CD4 binding site (8, 19, 20). The apparent importance of conformational epitopes as critical targets for neutralization of both HIV-1 and SIV suggests that the SIV-infected macaque model would make a

relevant system for dissection of the role of antibody in protection.

A number of immunoprophylaxis trials have been conducted with serum or plasma collected from SIV-infected animals but have yielded conflicting results. In two studies, infusion of a plasma pool from SIVmac-infected animals or a cocktail of four neutralizing mouse monoclonal antibodies (13, 24) failed to protect any of the recipient macaques from a homologous SIV challenge. However, two other trials reported partial protection against a homologous intravenous virus challenge following administration of a plasma pool from SIVsm- or SIVmne-infected macaques (29, 38) and one reported partial protection against a heterologous SIVsm/B670 challenge (9). Protection of two of three neonatal macaques from oral challenge as a result of transplacentally acquired SIV antibodies also suggests a beneficial role for antibodies (49). Finally, in a postexposure immunotherapy trial, administration of purified immunoglobulin G (IgG) (SIVIG) from an SIV-infected LTNP macaque had a beneficial effect in modifying subsequent disease progression (14). Unfortunately, the neutralizing antibodies in these various pools differed significantly in character and breadth and therefore the conflicting results of many of these trials cannot be resolved.

The SIV-infected macaque model offers a unique system for dissection of the antiviral antibody response at the molecular level and assessment of the role of protective epitopes by in vivo experimentation. For this reason, we initiated a study to produce SIV envelope monoclonal antibodies with high-level neutralizing activity that could serve as standardized, renewable reagents. Since the humoral immune response to SIV infection mounted by LTNP might be predictive of an effective immune response and based upon the beneficial clinical effect of purified SIVIG from such a macaque (14), this nonprogressor macaque was chosen as a source of immune cells to extract RNA for the purpose of generating a combinatorial phage display library. The present report describes the recovery by phage display technology of envelope-specific Fabs with high-level homologous neutralizing activity.

MATERIALS AND METHODS

Animal. An inguinal lymph node was biopsied from a rhesus macaque, E544, at 6 years post-SIVsmF236 challenge and was used as a source of mRNA for construction of a combinatorial phage display Fab library. This healthy monkey was also the source of a plasma pool used in a postexposure passive immunotherapeutic trial that demonstrated a beneficial effect of simian antibodies (14). The lymph node biopsy was gently disrupted and stored as a viable single-cell suspension in 10% dimethyl sulfoxide and 10% fetal calf serum in liquid nitrogen.

Construction of a lymph node γ1/κ antibody phage display library. Total RNA isolated from 1.3×10^7 lymph node cells (RNA Isolation Kit; Stratagene, La Jolla, Calif.) was reverse transcribed into cDNA by using an oligo(dT) primer. Thirty cycles of 94°C for 15 s, 52°C for 50 s, and 72°C for 90 s were performed. Macaque κ-chain genes were amplified with primers specific for human κ light-chain genes. The Fd segment (variable and first constant domains) of macaque heavy-chain genes was amplified with a combination of family-specific human VH primers based at the 5′ end and a macaque isotype γ1-specific primer based at the 3′ end (16, 40), as listed in Table 1. A total of 27 heavy-chain and 21 κ-chain amplification reactions, each with a single pair of primers, were conducted.

Purification, restriction digestion, and sequential ligation of light- and heavy-chain DNAs into the pCOMB3H phage display vector and transformation of electrocompetent *Escherichia coli* XL-1 Blue (Stratagene) were performed essentially as previously described (2, 7). Briefly, 450 ng of κ-chain DNA digested with *Sac*I and *Xba*I was ligated into 1,500 ng of pCOMB3H which had been digested with *Sac*I and *Xba*I, and the ligated product was transformed into *E. coli* XL-1 Blue. Transformants were propagated overnight at 37°C by solid-phase amplification (28) in 3 liters of 0.3% SeaPrep agarose (FMC BioProducts, Rockland, Maine) in TerrificBroth (Gibco/BRL, Gaithersburg, Md.) supplemented with 1% glucose, 50 μg of carbenicillin per ml, and 10 μg of tetracycline per ml. Phagemid DNA (κ-chain pCOMB3H) was isolated from this overnight culture. Nine hundred nanograms of κ1 chain digested with *Xho*I and *Spe*I was ligated into 3,000 ng of similarly digested κ-chain pCOMB3H, and the ligated product

was transformed into *E. coli* XL-1 Blue. Transformants were expanded in a volume of 5 liters by solid-phase amplification as described above. The final library of 3×10^7 clones was stored in 12.5% glycerol–Luria-Bertani (LB) broth at −80°C until use.

Panning and ELISA reagents. SIVsmH4 recombinant glycoprotein 130 (rgp130) expressed in CHO cells was a gift from Nancy Haigwood, Bristol-Myers Squibb Pharmaceutical Research Institute (37, 42). SIVmac251 rgp120 expressed in baculovirus was purchased from Intracel Inc. (Cambridge, Mass.), and the following reagents were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases: SIV-mac239 rgp140 produced by vaccinia virus provided by the Vaccine Research and Development Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases; HIV-2 ST rgp120 expressed in S2 drosophila cells from Margery Chaikin, SmithKline Beecham Pharmaceuticals (4, 17, 26); and mouse monoclonal antibodies KK45 and KK46, specific for the V3 loop of SIV gp120, from Karen Kent and Caroline Arnold (25). In all panning and enzyme-linked immunosorbent assay (ELISA) experiments, recombinant envelope antigens were diluted to 2 to 8 μg/ml in phosphate-buffered saline (PBS) and adsorbed to EIA/RIA A/2 (ELISA) plates (Costar, Cambridge, Mass.) overnight at 4°C.

Library screening. A total of 10^9 bacteria of the library stock were thawed and grown for 90 min at 37°C in 100 ml of $2 \times$YT broth (Gibco/BRL) supplemented with 1% glucose, 100 μg of carbenicillin per ml, and 10 μg of tetracycline per ml. Bacteria were pelleted and resuspended in 100 ml of $2 \times$YT broth with 100 μg of carbenicillin per ml and 10 μg of tetracycline per ml, and helper phage VCS M13 (Stratagene) was added at a multiplicity of infection of 50. The culture was expanded to 1,000 ml and shaken for 5 to 6 h at 37°C (30). Phage particles were precipitated overnight at 4°C from the clarified supernatant with 4% polyethylene glycol and 0.5 M NaCl, recovered by centrifugation, and resuspended in 2% skim milk powder in PBS with 0.05% sodium azide. ELISA wells were coated with the selection antigen, incubated overnight, and blocked with 2% skim milk powder in PBS and 0.05% sodium azide for 1 h at room temperature prior to addition of phage particles. Unbound phage particles were removed after 1 h by washing each well 10 times with PBS containing 0.05% Tween 20. Bound phage particles were eluted with 100 mM triethylamine (Sigma, St. Louis, Mo.) and immediately neutralized with 2 M Tris (pH 7.2). Exponentially growing *E. coli* XL-1 Blue was infected with the eluted phage and expanded by solid-phase amplification as described above. The eluted phage particles (clones) were enumerated after each round of panning and when a 5- to 10-fold increase was encountered (typically after the third round), phagemid DNA was extracted. The gene III-encoding segment of the phagemid was removed by restriction digestion with *Spe*I and *Nhe*I, and the phagemid DNA was self-ligated and transformed into *E. coli* XL-1 Blue. A total of 30 to 60 single colonies were picked, and each was inoculated into 200 μl of LB broth supplemented with 1% glucose and 100 μg of carbenicillin per ml in 96-well microtiter plates and grown overnight at 30°C. Fab production was induced by growing the colonies in 200 μl of LB broth with 100 μg of carbenicillin per ml and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Gold Biotechnology, St. Louis, Mo.) for 5 h. Crude bacterial supernatants were assayed directly by ELISA for reactivity with the same antigen used for selection.

Large-scale Fab production and purification. To facilitate purification of Fabs, a six-residue histidine tail (6×HIS) was added to the C terminus of the heavy chain by overlapping PCR. Primers 5′-CAG GTG CAG CTG CTC GAG TCG GG-3′ and 5′-TCC CAG ATC TGC GGC CGC TTA ATT AAT TTA ATG GTG ATG G-3′ were used to amplify the heavy-chain gene from Fab clone 201. The primary PCR product was reamplified with primers 5′-CAG GTG CAG CTG CTC GAG TCG GG-3′ and 5′-TAA TTA ATG GTG ATG GTG ATG GTG GCT AGC ACC ACC ACA TGT TTT G-3′. The resulting fragment was cut with restriction enzymes *Xho*I and *Not*I and ligated back into Fab clone 201 which had been digested with *Xho*I and *Not*I to create Fab 201-6×HIS. Fab clone 201-6×HIS was subsequently used as a cloning vector to introduce 6×HIS tails into Fab clones 101, 102, 202, 203, and 204 by subcloning the *Sac*I-to-*Sau*I fragment.

Bacterial cultures of 1 to 2 liters were grown in LB broth (Gibco/BRL) with 1% glucose and 100 μg of carbenicillin per ml at 30°C. At an optical density at 600 nm of approximately 0.2, the bacteria were pelleted and resuspended in LB broth supplemented with 100 μg of carbenicillin per ml and 0.1 mM IPTG. Cultures were induced for 5 h at room temperature. For Fabs with a 6×HIS tail, the bacterial pellet was resuspended in 20 to 40 ml of PBS–0.1 mM phenylmethylsulfonyl fluoride and subjected to three cycles of freezing (−80°C) and thawing (37°C). The extract was clarified by centrifugation and purified by chelate chromatography on a nitrilotriacetic acid column (Novagen, Madison, Wis.) by following the supplier's instructions. For Fabs without a 6×HIS-tail, the bacterial pellet was resuspended in 20 to 40 ml of 20 mM Tris (pH 8.6) and subjected to three cycles of freezing (−80°C) and thawing (37°C). The clarified extract was applied to a 10-ml open DEAE–Affi-Gel Blue column (Bio-Rad, Hercules, Calif.) and washed with 20 column volumes of 20 mM Tris (pH 8.6). The column was eluted stepwise in 20-ml fractions containing 100, 250, and 500 mM NaCl. All fractions were tested in an ELISA for reactivity with antigen. Most macaque Fabs eluted in the 100 mM NaCl fraction when tested by ELISA for gp120 reactivity. Purified Fab was concentrated and dialyzed against PBS. Fab purity was judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and concentration was determined with a BCA Protein Assay Reagent kit (Pierce,

Rockford, Ill.) with bovine serum albumin as the standard. The concentration of DEAE–Affi-Gel Blue-purified Fabs was estimated with an antigen capture ELISA using unconjugated and alkaline phosphatase-conjugated goat anti-human Fab antibodies (Pierce). Nitrilotriacetic acid column-purified Fab served as a standard.

ELISA analysis of Fab reactivity and cross-reactivity. The following protein antigens were diluted and adsorbed to polystyrene plates as described above: SIV or HIV-2 gp120 at 2 µg/ml, carbonic anhydrase at 10 µg/ml, ovalbumin at 10 µg/ml, and thyroglobulin (Sigma) at 100 µg/ml. Plates were blocked with 3% bovine serum albumin–PBS for 1 h, and 50 µl of crude or purified Fab was subsequently added to the wells. Following 1 h of incubation, Fab binding was detected with a 1:1,000 goat anti-human F(ab′)$_2$-alkaline phosphatase conjugate (Pierce). The assay was developed with 1 mg of *p*-nitrophenyl phosphate (Sigma) per ml.

Epitope mapping. In initial experiments, Fabs 102 and 202 were tested in a competition ELISA as described previously (23). A panel of mouse monoclonal antibodies that define four epitope clusters on the SIV envelope glycoprotein were used: a conformation-dependent neutralization epitope in the V3-V4 region (KK5, KK9, KK17, KK44, KK56, and KK57), a linear epitope in the V3 loop (KK45 and KK46), a linear epitope in the V1-V2 region (KK13), and an epitope within gp41 (KK41). In a second series of experiments, the epitope specificities of Fabs 101, 102, 103, 201, 202, 203, and 204 were determined by using KK45 and 201-IgG1. Antibody 201-IgG1 is Fab 201 converted into a whole macaque IgG1 and expressed in COS cells (unpublished data). Subsaturating concentrations of KK45 and 201-IgG1 were mixed with serial dilutions of Fabs. The mixture was transferred to plates coated with SIVmac251 gp120 at 2 µg/ml and incubated for 2 h. Binding of mouse monoclonal antibody and 201-IgG1 was detected with a goat anti-mouse Ig-alkaline phosphatase conjugate (Jackson ImmunoResearch, West Grove, Pa.) and a goat anti-human IgG (heavy and light chains)-alkaline phosphatase conjugate, respectively. The latter conjugate does not cross-react with macaque Fabs expressed in bacteria; thus, it only detects whole macaque IgG. Fab binding was measured in parallel by setting up duplicate dilution series.

Peptide mapping. Twenty-mer peptides, with an overlap of 10 amino acids, spanning the whole of SIVmac251 gp120 (EVA774) were obtained from the Reagent Program of the European Vaccine against AIDS. The reactivity of Fab 102 against these peptides was tested in an ELISA.

Assay of SIV-neutralizing antibodies. SIV neutralization was measured by using a cell-killing assay as previously described (15, 32). All neutralization experiments were performed with purified Fabs or heat-inactivated plasma samples. SIVsmH4, SIVsmE543-3, and SIVmac239 are molecularly cloned viruses, whereas SIVsmE660, SIVsmB670, and SIVmac251 are uncloned virus stocks. Virus stocks were produced in H9 cells (SIVsmH4, SIVsmB670, and SIV-mac251) or CEM×174 cells (SIVsmE543-3 and SIVmac239).

Nucleic acid sequencing and analysis. Nucleic acid sequencing was carried out on a 373A automated DNA sequencer (Applied Biosystems) using a *Taq* fluorescent dideoxynucleotide terminator cycle sequencing kit. The following sequencing primers were used: heavy chain, 5′-ATTGCCTACGGCAGCCGCTGG-3′ (HC1) and 5′-GGAAGTAGTCCTTGACCAGGC-3′ (HC4); κ chain, 5′-ACAGCTATCGCGATTGCAGTG-3′ (LC1) and 5′-CACCTGATCCTCAGATGGCGG-3′ (LC4). Resulting sequences were analyzed by using MacVector (International Biotechnologies, New Haven, Conn.) and GeneWorks (IntelliGenetics, Campbell, Calif.) software. Sequence similarity searches were performed with V-base, a compilation of all available human variable-segment Ig germ line sequences obtained from I. Tomlinson (10).

RESULTS

Macaque SIV gp120-specific Fabs. RNA extracted from an inguinal lymph node of SIV-infected LTNP rhesus macaque E544 was used as the template for first-strand cDNA synthesis. With the primers shown in Table 1, the κ light-chain gene and the Fd segment of the γ1 heavy-chain gene were amplified and cloned into a phagemid vector. The resulting Fab phage library was then selected against monomeric gp120 preparations of SIVsmH4, SIVmac251, or HIV-2/ST, and seven SIV gp120-specific Fabs were isolated. Every clone, except Fab 204, was recovered at least twice in independent panning experiments, showing the reproducibility of the propagation procedure employed in this study. To increase the diversity of the Fabs recovered or enrich for rarer Fabs, two epitope-masking experiments were conducted. Masking of the epitope recognized by Fab 201 resulted in the reisolation of group 1 clones solely. The masking of two epitopes (Fabs 101 and 201) resulted in the isolation of a variety of clones of which only one was new, Fab 204. In addition, two Fabs (1LNRhE544 and 2LNRhE544) were selected from the unscreened library and used as negative controls in subsequent experiments.

TABLE 1. Summary of oligonucleotide primers used to amplify the Fab library

| Primer(s)	Sequence
5′ primers with *Sac*I site-kappa light chain	5′-GACATCGAGCTCACCCAGTCTCTCCA-3′
	5′-GACATCGAGCTCACCCAGTCTCCA-3′
	5′-GATATTGAGCTCACTCAGTCTCCA-3′
	5′-GAAATTGAGCTCACGCAGTCTCCA-3′
	5′-GAAATTGAGCTCAC(G/A)CAGTCTCCA-3′
	5′-GAGCCGCACGAGCCCGAGCTCCAGATGACCCAGTCTCC-3′
	5′-GAGCCGCACGAGCCCGAGCTCGTTG(A/T)TGAC(A/G)CAGTCTCC-3′
3′ primer with *Xba*I site-kappa light chain	5′-GCGCCGTCTAGAATTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGAACTCAG-3′
5′ primers with *Xho*I site-heavy chain	5′-CAGGTGCAGCTCGAGCAGTCTGGGG-3′
	5′-CAGGTGCAGCTGCTCGAGTCTGGGG-3′
	5′-CAGGTGCAGCTACTCGAGTCGGGG-3′
	5′-GAGGTGCAGCTCGAGGAGTCTGGGG-3′
	5′-GAGGTGCAGCTGCTCGAGTCTGGGG-3′
	5′-CAGGTCAGCTGCTCGAGTCAGG-3′
	5′-AGTCGCAGCTGCTCGAGTCTGAGG-3′
	5′-AGTGCAGCTGCTCGAGTCTGGG-3′
	5′-CAGGTGCAGCTGCTCGAGTCGGGG-3′
	5′-CAGGTGCAGCTACTCGAGTGGGGG-3′
3′ primer with *Spe*I site-heavy chain	5′-AGGTTTACTAGTACCACCACATGTTTGATCTC′-3′
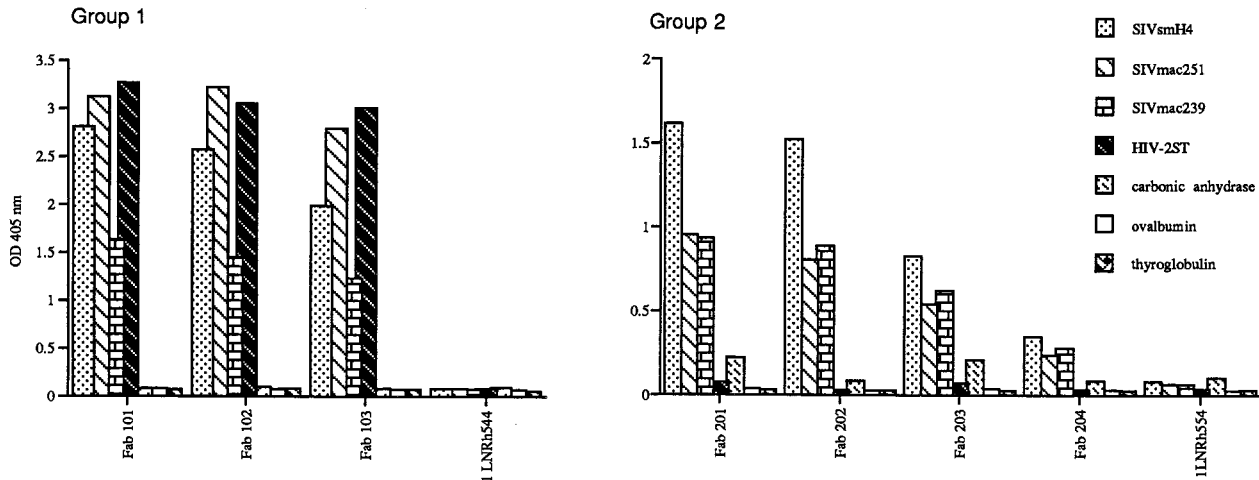
FIG. 1. Graphical representation of ELISA results. Reactivity of Fabs (10 µg/ml) to monomeric rgp120 from various SIV and HIV-2 isolates and a panel of irrelevant protein antigens. Fabs 101, 102, 103, 201, 202, 203, and 204 were selected by panning of the library against monomeric SIV rgp120, whereas 1LNRh544 is a randomly picked clone from the unscreened library.

SIV gp120-specific Fabs form two distinct groups. The specificity of the selected Fabs was assessed by ELISA using a panel of SIV and HIV-2 recombinant envelope proteins. As shown in Fig. 1, all seven Fabs were envelope specific, with no evidence of cross-reactivity to a panel of randomly chosen, unrelated protein antigens. Three of the Fabs were broadly reactive, binding to recombinant gp120 from SIVsmH4, SIVmac251, SIVmac239, and HIV-2/ST, whereas the reactivity of the other four Fabs was restricted to the SIV envelope preparations and did not include HIV-2 rgp120, suggesting that two distinct groups of Fabs had been recovered. Significantly, one of the Fabs with broad reactivity had been obtained by panning with HIV-2/ST gp120. Based upon this binding specificity, the Fab clones were numbered according to these two groups as 101,

102, and 103 for the broadly reactive Fabs and 201 to 204 for those specific for SIV envelopes alone.

Sequence analysis confirmed the grouping based upon antigen binding. As shown in Fig. 2, the deduced amino acid sequence of the VH-D-JH and Vκ-Jκ segments illustrated relatedness among the Fabs within each group. Most notably, the heavy-chain CDR3 (HCDR3) regions within a group were conserved in terms of amino acid sequence and length. Within group 1, the heavy-chain sequences of Fabs 101, 102, and 103 shared 77 to 87% identity overall, with the greatest divergence observed in CDR2, FR3, and CDR3. The κ chains of clones 101 and 102 were identical, with the exception of two amino acid differences at the amino terminus due to the use of different primers for amplification. The κ chain of clone 103 was

Heavy chain amino acid sequences - variable region

κ light chain amino acid sequences - variable region

FIG. 2. Alignment of the deduced amino acid sequences of the variable domains of the heavy and κ chains. Substitutions relative to either Fab 101 or 201 are shown in single amino acid code. Identical residues are indicated by dashes. Amino acids are numbered in accordance with Kabat et al. (21), and complementary determining regions (CDR1, CDR2, and CDR3) and framework regions (FR1, FR2, FR3, and FR4) are shown above the sequence alignments.

```
              VH    |    D
            _____|_____
Fab 101     TGT GCG ACT CTG TCG TAC
Fab 102     --- --- -G- --- -AC --T
Fab 103     --- --- -G- --- --C --T


Fab 201     TGT GCG AGG GAT CGT GTA
Fab 202     --- --- --- --- --- ---
Fab 203     --- --- --A --- -T- A-T
Fab 204     --- --- --A --- --C T-T
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FIG. 3. Alignment of the nucleotide sequences of the VH-D junctions from various SIV rgp120-specific Fab clones. Three codons on either side of the VH-D junction of Fab 101 or 201 are shown. Substitutions in clones 102, 103, 202, 203, and 204 are indicated by the appropriate nucleotides, and identity is indicated by dashes.

TABLE 2. Homology of macaque heavy and kappa light chains of various SIV-specific Fabs with human Ig germ line genes[a]

Fab clone	V_H	D	J_H	V_K	J_K
101	4.39	ND	J4b	DPK4	JK1
102	4.39	ND	J4b	DPK4	JK1
103	4.39	ND	J4b	DPK4	JK1
201	4.42	ND	J6b	DPK18	JK1
202	4.42	ND	J6b	DPK18	JK1
203	DP-67	ND	J6b	DPK18	JK1
204	DP-71	ND	J6b	DPK18	JK1

[a] The human germ line segment with the highest score by DNA matrix analysis in MacVector is identified. ND, not determined due to lack of any identifiable homolog.

only slightly more divergent, differing from the latter two clones by six amino acid substitutions.

Within group 2, Fabs 201 and 202 were highly related, differing in only nine residues in the heavy chain and one residue in the kappa chain (due to the use of a different PCR primer). These clones had identical HCDR3 sequences. Fab 204 was the most divergent clone within this group, differing in 36 residues from either Fab 201 or 202. Despite the overall divergence, each of the Fabs within this group showed remarkably little variation in the HCDR3; most of the amino acid differences were seen within HCDR1, HCDR2, and FR3. An additional unique feature of each of the group 2 Fabs was the presence of two cysteine residues in HCDR3 that probably are bridged by a disulfide bond, thus forming an extra loop.

The striking length and sequence uniformity of the CDR3 regions within each group of Fabs suggested that each group might represent somatic variants of a common precursor. However, there were a number of differences at the nucleotide level at the VH-D junctional regions of the group 1 Fabs (Fig. 3), despite conserved amino acid sequences, indicating that the clones probably arose from independent recombinational events. In group 2, Fabs 201 and 202 had identical VH-D junctional regions and were probably somatic variants, but Fabs 203 and 204 appeared distinct and also may have arisen from independent precursors.

The occurrence of specific Fabs with relatively conserved HCDR3 regions but considerable variability throughout the rest of the VH gene has been described previously for anti HIV-1 gp41 Fabs (5). This could arise from multiple crossover events occurring during PCR amplification of antibody genes in library construction. A more likely explanation is that it represents convergent evolution driven by an HCDR3 motif giving high-affinity binding to a molecular feature on gp120.

To tentatively establish the germ line origin of the Fab clones, we relied upon the extensive homology between macaque and human Igs to conduct similarity searches of all known human Ig genes. As shown in Table 2, all of the heavy-chain sequences exhibited homology to the human VH4 family of germ line segments. All of the group 1 Fabs were most related to VH4.39. The heavy chains of Fabs 201 and 202 showed the best match to human VH segment 4.42 (50), whereas Fabs 203 and 204 showed the best alignment to human VH segments DP-67 (51) and DP-71 (47), respectively. However, in all four homology searches, a high score was obtained for human VH segment DP-67. Significant similarity to any of the human D segments was not found by searching V-base or all of GenBank.

Macaque Fabs react with two distinct regions of SIV gp120. To identify the epitopes recognized by these macaque Fabs, we tested their ability to compete for binding in an ELISA with

mouse monoclonal antibodies of known specificity. We utilized a series of mouse monoclonal antibodies that were recovered by Kent et al. following immunization with gp120 of SIVmac (23, 25), which shares 82% identity with SIVsm, the strain that induced the Fabs. As seen in Fig. 4a, Fab clone 102 competed with the binding of MAbs KK45 and KK46, which both recognize a linear epitope in the V3 loop. Peptide mapping indicated that Fab 102 reacted with two overlapping peptides (EVA774.30 and EVA774.31 (1) in the V3 loop (data not shown). Thus, it appeared that the epitope recognized by Fab 102 was contained within 10-mer peptide VTIMSGLVFH (amino acids 322 to 331), of which the last seven residues are perfectly conserved in SIVmac, SIVsm, and HIV-2, thereby accounting for the cross-reactivity of this Fab with these three envelope preparations. As expected based on sequence similarity, each of the group 1 Fabs also competed with V3 loop-specific monoclonal antibody KK45 (Fig. 4c). In contrast, Fab 202 competed with two neutralizing monoclonal antibodies, KK5 and KK9 (Fig. 4b), that target another epitope cluster (KK5, KK9, KK17, KK44, KK56, and KK57) (22, 23, 25) and recognize a conformation-dependent epitope in the V3-to-V4 region of the SIV envelope (8). Whole antibody 201-IgG1 inhibited the binding of all group 2 Fabs, consistent with recognition of a common region by these Fabs (Fig. 4d).

Neutralizing activity of macaque Fabs. All seven SIV-specific Fabs, including the two randomly chosen Fabs from the unscreened library, were purified and tested for neutralizing activity in vitro. Only Fabs 201 and 202 demonstrated neutralizing activity against the homologous molecularly cloned virus SIVsmH4, as illustrated in Fig. 5. Although related to these clones, Fab clones 203 and 204 did not exhibit neutralizing activity. Subsequently, Fabs 201 and 202 were evaluated against a broader panel of viruses belonging to the SIVsm and SIVmac lineage. As shown in Table 3, the homologous virus SIVsmB670 was readily neutralized by Fabs 201 and 202 at a concentration of about 40 ng/ml, whereas heterologous viruses such as SIVmac251 and SIVmac239 were refractory to neutralization in vitro. A recently characterized neutralization-resistant infectious clone, SIVsmE543-3 (15), that also belongs to the SIVsm lineage, and shares 92% envelope identity with SIVsmH4, was resistant to neutralization by Fab 201 or 202 (data not shown). The type specificity of neutralization by these macaque Fabs was similar to the neutralizing profile observed for SIVIG purified from this donor macaque; the polyclonal SIVIG neutralized homologous SIVsmE660 and SIVsmB670 at a concentration of approximately 1 μg/ml but required a 40-fold higher concentration to neutralize the heterologous virus SIVmac251 (Table 3).

a.   Fab 102

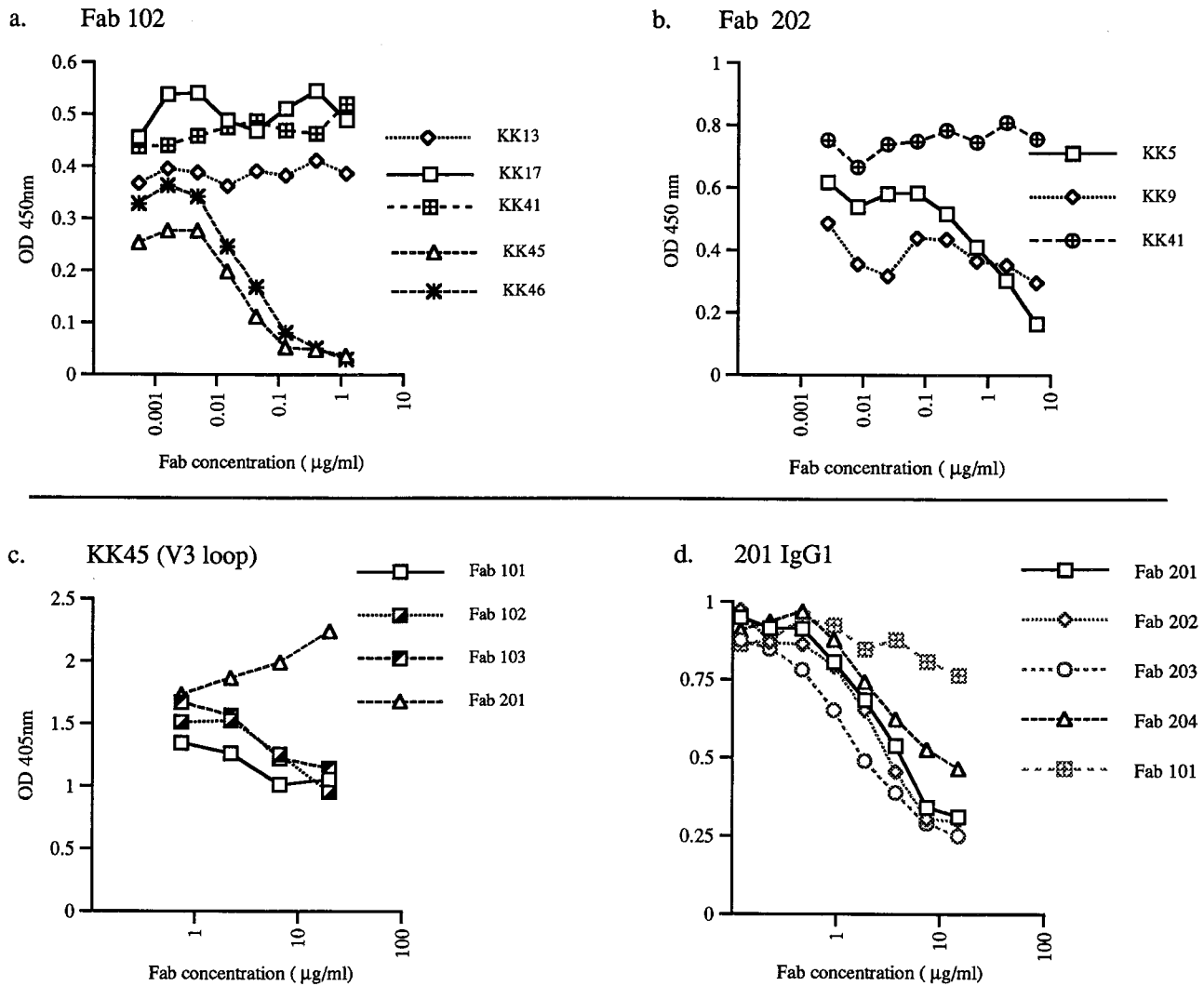b.   Fab 202

c.   KK45 (V3 loop)

d.   201 IgG1

FIG. 4. Identification of epitope specificity of macaque Fabs by competition ELISA with mouse monoclonal antibodies of defined specificity. Competition for binding of mouse monoclonal antibodies with a dilution series of Fab 102 (a) and Fab 202 (b) as the competitor. (c) Competition of Fabs 101, 102, 103, and 104 with V3 loop-specific monoclonal antibody KK45 as measured by the binding of the mouse monoclonal antibody. (d) Competition of Fabs 201, 202, 203, and 204 with antibody 201-IgG1 as measured by binding of the whole macaque antibody. OD, optical density.

DISCUSSION

This study demonstrates the usefulness of the antibody phage display library approach for recovering antigen-specific macaque antibodies from a SIV-infected animal and describes the first fully homologous macaque Fabs to be generated by this technique. In the present study, seven unique SIV gp120-specific macaque Fabs were obtained by screening a lymph node cDNA library from a healthy long-term survivor of SIV infection (rhesus macaque E544). Based on nucleotide sequence similarity, pattern of gp120 reactivity, and epitope-mapping analysis, as defined by competition with previously characterized mouse monoclonal antibodies, the Fab clones formed two distinct groups. One group of Fabs recognized a nonneutralization, linear epitope in the V3 loop, whereas the other recognized a neutralization, conformation-dependent epitope in the V3-to-V4 region of gp120. Neutralizing Fabs 201 and 202 were highly effective in neutralizing virus isolates very closely related to the virus that had infected this macaque (SIVsmH4 and SIVsmB670) but failed to neutralize the het-

erologous virus SIVmac251. Not unexpectedly, the Fabs also failed to neutralize a homologous, molecularly cloned, neutralization-resistant SIVsm (SIVsmE543-3 [15]). These findings underscore the observation that a biologically important neutralization epitope recognized by SIV-infected animals is within the V3-to-V4 region (8, 20, 22, 23, 25).

The specificity of binding of the Fabs to various gp120 preparations was not predictive of the ability to neutralize the isolates from which these envelopes were cloned. For example, group 2 Fabs bound to SIVmac and SIVsm gp120 preparations but neutralized only SIVsm isolates. This disparity between the ability to bind monomeric gp120 and neutralization might be due to differences in affinity for the oligomeric envelope as expressed on virions. This disparity has precedents in other viral systems, including HIV-1. For example, affinity of monoclonal antibodies for the mature oligomeric form of the envelope glycoprotein is more predictive of neutralization of HIV-1 primary isolates than is binding to monomeric forms of this envelope antigen (12, 33, 39, 41, 46). Interestingly, the prefer-
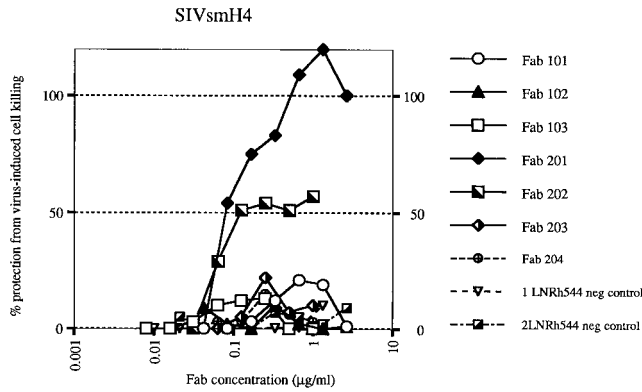
FIG. 5. Neutralization titration curve of macaque Fabs against SIVsmH4. The Fab concentration is plotted versus the resulting protection from virus-induced killing in CEM×174 cells. Two Fabs (201 and 202) neutralized SIV in a dose-dependent fashion, whereas all other Fabs, including two irrelevant Fabs, 1LNRh544 and 2LNRh544 (negative [neg] controls); from the unscreened library, failed to neutralize SIV, even at high concentrations.

ential ability of the Fabs to neutralize homologous isolates was similar to the profile of a contemporaneous IgG preparation purified from this macaque, suggesting that the neutralizing Fabs recovered from this combinatorial library were representative of the antibody response of this animal. Although this library is unlikely to represent the full antibody repertoire, this technique shows promise as a method for the recovery of macaque SIV-specific neutralizing Fabs. Potential factors that could limit the repertoire are the restricted size of the library (3×10^7 clones) and the exclusive use of kappa light chains and γ1 heavy chains. Finally, since this is a systemic infection, the actual choice of tissue for generation of the library could affect the overall representation of clones. However, in an independently screened bone marrow library prepared from the same animal, all but one of the Fab clones grouped both biologically and genetically with those obtained from the lymph node library (data not shown), suggesting that the tissue source was not a major determinant of the antibody repertoire representation.

In summary, the SIV envelope Fabs generated from a SIV-infected LTNP provide further insight into the epitopes recognized by such animals. Only two epitopes were recognized by this particular animal, one of which constituted a neutralization epitope. A third Fab generated from the bone marrow library recognized a third epitope in the V1 region of gp120 which did not induce neutralizing antibodies (data not shown). This animal exhibited tightly restricted viral replication and, perhaps as a consequence, had a low-to-moderate serum neu-

tralizing antibody titer which was fairly type specific. The spectrum of epitopes recognized by sera of SIV-infected progressor macaques may be more extensive. We are currently generating IgG1 macaque monoclonal antibodies which express the representative Fabs of each group. Such macaque monoclonal antibodies should prove valuable in defining the role of neutralizing antibody in preventing or ameliorating SIV infection in vivo. Additionally, the availability of molecularly cloned SIV stocks that are sensitive or resistant to neutralization by this antibody will facilitate studies intended to define the in vivo role of neutralizing antibodies that are identified by in vitro assays.

TABLE 3. Profile of neutralizing activities of Fabs 201 and 202 against various SIV isolates[a]

Prepn	Concn (µg/ml) of Fab or IgG needed to neutralize indicated SIV strain				
	Homologous strains			Heterologous strains	
	smH4	smB670	smE660	SIVmac251	SIVmac239
Fab 201	0.078	<0.042	ND	>5.53	>5.53
Fab 202	0.117	0.038	ND	>2.00	>2.00
SIVIG	ND	1.35	0.78	40.4	>50.0

[a] The concentrations of Fab or SIVIG that resulted in 50% protection of CEM×174 cells from virus-induced cell killing are shown. ND, not determined.

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