and triisopropylsilane (8.15/0.75/0.75/0.25/0.25/0.1, v/v). After removal of the resins by filtration, the filtrate was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethyl ether. All crude peptides were purified by RP-HPLC and identified by ESI-TOFMS. In the conjugation of the R_8 peptide (or iodoacetamide), the peptide (or iodoacetamide) solution in 0.1 M phosphate buffer, pH 7.8 was added to MA fragments which were synthesized as described above. The reaction mixture was stirred at room temperature under nitrogen. After 24 h (or 1 h for the conjugation of iodoacetamide), purification was performed by RP-HPLC. The purified peptides were identified by ESI-TOF MS and lyophilized. Purities of all final compounds were confirmed to be >95% by analytical HPLC. Detailed data are provided in Supplementary data.

2.2. Anti-HIV-1 assay

Anti-HIV-1 (NL4-3 or NL(AD8)) activity was determined by measurement of the protection against HIV-1-induced cytopathogenicity in MT-4 cells or PM1/CCR5 cells. Various concentrations of test peptide solutions were added to HIV-1 infected MT-4 or PM1/CCR5 cells at multiplicity of infection (MOI) of 0.001 and placed in wells of a 96-well microplate. After 5 day incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The anti-HIV-1 (JR-CSF) activity was also determined by measuring capsid p24 antigen concentrations of the culture supernatant in the infected cultures by a commercially available ELISA assay (ZeptoMetrix Corp., Buffalo, NY).

2.3. CD spectroscopy

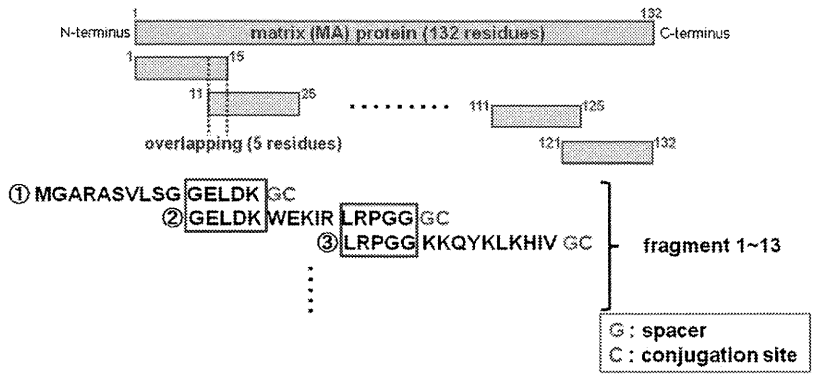
CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C. The measurements were performed using a 0.1 cm path length cuvette at a 0.1 nm spectral resolution. Each spectrum represents the average of 10 scans, and the scan rate was 50 nm/min. The concentrations of samples 8L and 9L were 28.2 and 64.7 μM, respectively, in PBS buffer (pH 7.4).

2.4. Fluorescent imaging of cell-penetrating MA peptides

Cells were seeded on 35 mm glass-bottom dish (2×10^5 cells/dish for HeLa and A549, 1×10^5 cells/dish for CHO-K1) one day before the experiments. The cells were cultured in DMEM/10% FBS/Penicillin–Streptomycin for HeLa and A549, or Ham's F12/10% FBS/Penicillin–Streptomycin for CHO-K1 at 37 °C/5% CO_2. Before the addition of MA peptides, cells were washed with Hanks' balanced salt solutions (HBSS) once. Peptides were added at 5 μM and further cultured for 30 min at 37 °C/5% CO_2. After incubation, cells were washed three times with HBSS and observed under a confocal laser-scanning microscopy (Zeiss LSM510).

3. Results and discussion

An overlapping peptide library spanning the whole sequence of the MA domain, p17, of NL4-3, the Gag precursor Pr55 of HIV-1 was designed. The full sequence of MA consists of 132 amino acid residues. In the peptide library, the MA sequence was divided from the N-terminus in 15-residue segments with an overlap of 5



fragment number	sequence
1	H-MGARASVLSGGELDKGC-NH_2
2	CH_3CO-GELDKWEKIRLRPGGGC-NH_2
3	CH_3CO-LRPGGKKQYKLKHIVGC-NH_2
4	CH_3CO-LKHIVWASRELERFAGC-NH_2
5	CH_3CO-LERFAVNPGLLETSEGC-NH_2
6	CH_3CO-LETSEGSRQILGQLQGC-NH_2
7	CH_3CO-LGQLQPSLQTGSEELGC-NH_2
8	CH_3CO-GSEELRSLYNTIAVLGC-NH_2
9	CH_3CO-TIAVLYSVHQRIDVKGC-NH_2
10	CH_3CO-RIDVKDTKEALDKIEGC-NH_2
11	CH_3CO-LDKIEEEQNKSKKKAGC-NH_2
12	CH_3CO-SKKKAQQAAADTGNNGC-NH_2
13	CH_3CO-DTGNNSQVSQNYGC-NH_2

Figure 1. The construction of MA-based overlapping peptide library.

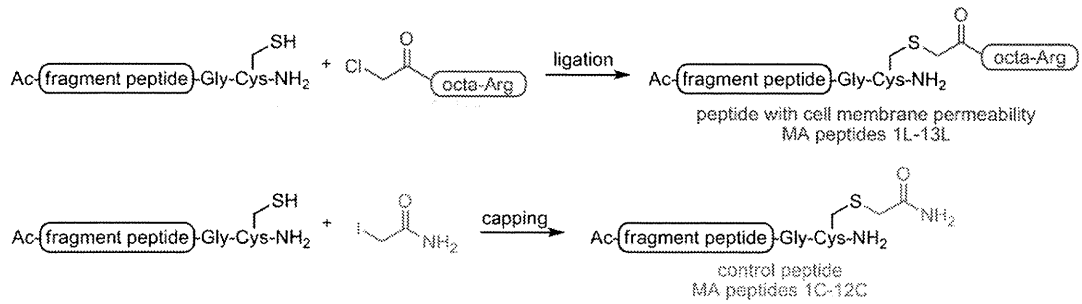


Figure 2. The design of MA peptides with cell membrane permeability (upper) and their control peptides (lower).

residues to preserve secondary structures (Fig. 1). Cys residues of the original MA sequence were changed into Ser residues because of the facility of peptide synthesis. Thirteen MA fragment peptides (1–13) were designed with the addition of Gly as a spacer and Cys as a conjugation site at the C-terminus. To impart cell membrane permeability to these peptides, the N-terminal chloroacetyl group of an octa-arginyl (R_8) peptide[19] was conjugated to the side-chain thiol group of the Cys residue of the above peptides. This resulted in the MA peptides 1L–13L (Fig. 2). R_8 is a cell membrane permeable motif and its fusion with parent peptides is known to produce bioactive peptides with no significant adverse properties.[12,13,20–24] In addition, the R_8-fusion can increase the solubility of MA

Table 1
Anti-HIV activity and cytotoxicity of control MA peptides

MA peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3 (MTT assay) EC_{50}[a] (μM)	NL(AD8) (MTT assay) EC_{50}[a] (μM)	JR-CSF (p24 ELISA) EC_{50}[a] (μM)	(MTT assay) CC_{50}[b] (μM)
1C	>50	ND	ND	>50
2C	17 ± 1.4	1.0	ND	>50
3C	>50	ND	ND	>50
4C	No inhibition at 12.5 μM	ND	ND	14
5C	>50	ND	ND	>50
6C	37 ± 12	24% inhibition at 6.25 μM	25% inhibition at 50 μM	>50
7C	>50	ND	ND	>50
8C	>50	ND	ND	>50
9C	29 ± 1.4	13	8.1	>50
10C	No inhibition at 12.5 μM	ND	ND	17
11C	>50	ND	ND	>50
12C	>50	ND	ND	>50
14C	>50	ND	ND	>50
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells and R5-HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM1/CCR5 cells evaluated by the MTT assay, and inhibitory activity against R5-HIV-1 (JR-CSF strain)-induced cytopathogenicity in PM1/CCR5 cells evaluated by the p24 ELISA assay.

[a] EC_{50} values are the concentrations for 50% protection from HIV-1-induced cytopathogenicity in MT-4 cells.

[b] CC_{50} values are the concentrations for 50% reduction of the viability of MT-4 cells. All data are the mean values from at least three independent experiments. ND: not determined.

Table 2
Anti-HIV activity and cytotoxicity of MA peptides with cell membrane permeability

MA peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3(MTT assay) EC_{50} (μM)	NL(AD8)(MTT assay) EC_{50} (μM)	JR-CSF(p24 ELISA) EC_{50} (μM)	(MTT assay) CC_{50} (μM)
1L	30	30	40	>50
2L	21 ± 4.2	>31	ND	32 ± 4.2
3L	no inhibition at 25 μM	ND	ND	36
4L	no inhibition at 3.13 μM	ND	ND	3.7
5L	40	42% inhibition at 50 μM	42	>50
6L	40 ± 8.9	49% inhibition at 50 μM	31	>50
7L	35 ± 1.5	37% inhibition at 50 μM	35% inhibition at 50 μM	>50
8L	2.3 ± 0.3	5.8	7.8	9.0 ± 2.4
9L	2.1 ± 0.5	0.43	0.58	5.7 ± 2.1
10L	43 ± 8.5	42% inhibitionat 50 μM	27	>50
11L	18 ± 3.0	17% inhibition at 25 μM	23	>50
12L	41 ± 5.5	30% inhibition at 25 μM	27	>50
13L	20 ± 2.1	0.43	11	>50
14L	no inhibition at 25 μM	ND	ND	36
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

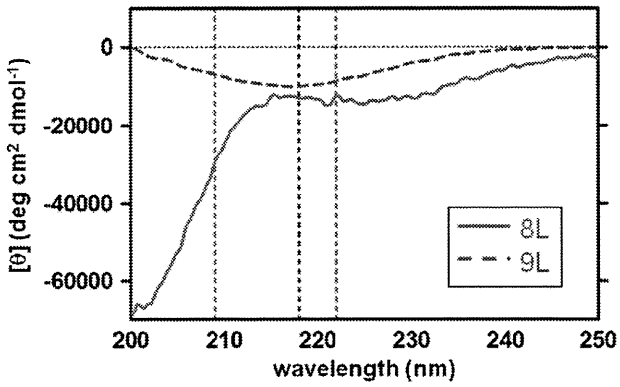


Figure 3. CD spectra of MA peptides 8L (28 μM) and 9L (65 μM) in PBS buffer, pH 7.4 at 25 °C.

peptides whose hydrophobicity is relatively limited. On the other hand, to develop control peptides lacking cell membrane permeability, iodoacetamide was conjugated to the thiol group of the Cys residue to prepare MA peptides 1C–12C (Fig. 2). MA peptide 13C was not synthesized because MA fragment 13 is insoluble in PBS buffer.

The anti-HIV activity of MA peptides 1L–13L and MA peptides 1C–12C, was evaluated. Inhibitory activity against T-cell line-tropic (X4-) HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells and against macrophage-tropic (R5-) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM1/CCR5 cells was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and inhibitory activity against R5-HIV-1 (JR-CSF strain) replication in PM1/CCR5 cells was determined by the p24 ELISA assay. The results are shown in Tables 1 and 2. The control MA peptides 6C and 9C showed slight anti-HIV activity against NL4-3, NL(AD8) and JR-CSF strains, and 2C showed high anti-HIV activity against NL4-3 and NL(AD8) strains, but the other control MA peptides showed no significant anti-HIV activity. 2C showed significant anti-HIV activity against both X4-HIV-1 and R5-HIV-1 strains, suggesting that this region of the MA domain is relevant with Gag localization to the plasma membrane (PM)[25] and that 2C might inhibit competitively the interaction between MA and PM. On the other hand, the MA peptides with the exception of 3L and 4L, showed moderate to potent anti-HIV activity against all three strains. These peptides expressed almost the same level of anti-HIV activity against both X4-HIV-1 and R5-HIV-1 strains. The MA peptides 8L and 9L in particular, showed significant anti-HIV activity. These results suggest that MA peptides achieve entry into target cells as a result of the addition of R_8, and inhibit viral replication within the cells. The adjacent peptides 8L and 9L possess an overlapping sequence TIAVL. Such peptides exhibited relatively high cytotoxicity and the MA peptide 4L showed the highest cytotoxicity although it did not show any significant anti-HIV activity. The control MA peptides 1C–12C were relatively weakly cytotoxic. The MA peptides 8C and 9C exhibited no significant cytotoxicity, although the addition of R_8, giving 8L and 9L, caused a remarkable increase in cytotoxicity. This suggests that the octa-arginyl (R_8) sequence is correlated with the

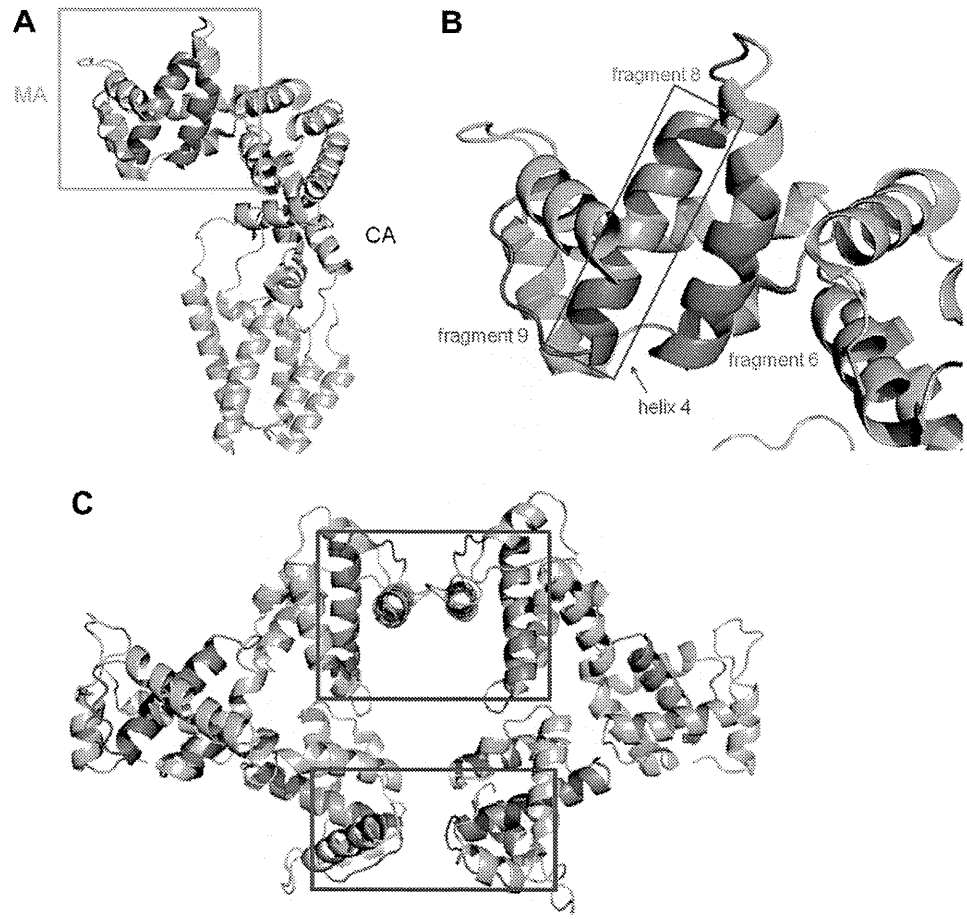


Figure 4. (A) The complete structure of MA and CA proteins (PDB ID: 2gol). (B) The enlarged structure of the highlighted region of (A). (C) The structure of an MA hexamer. Red-colored squares show interfaces between two MA trimers (PDB ID: 1hiw). Orange- and pink-colored helical ribbons represent fragments 8 and 9, respectively.

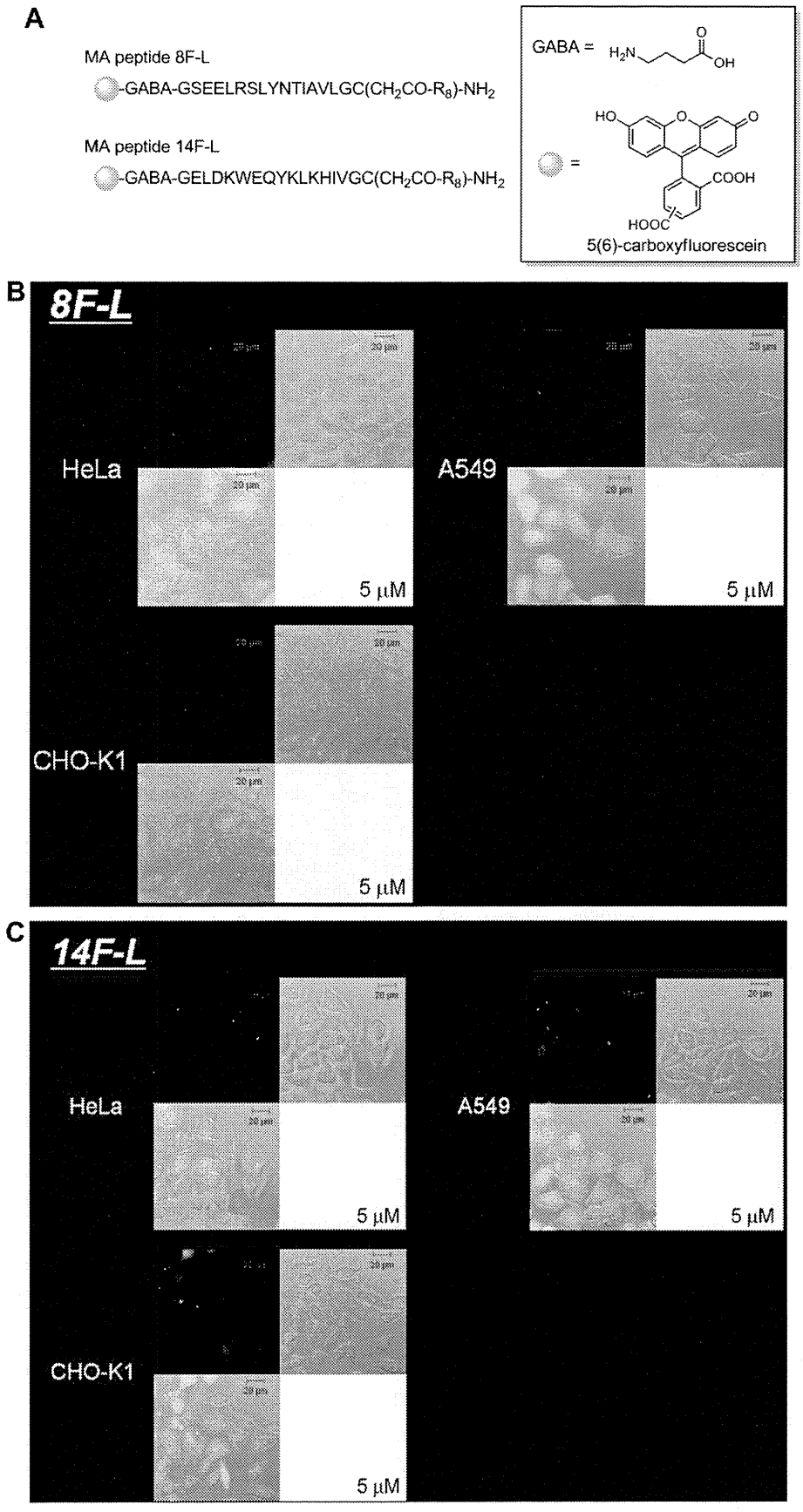


Figure 5. (A) The structures of fluorophore-labeled MA peptides 8F–L and 14F–L. (B) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 8F–L. (C) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 14F–L.

expression of cytotoxicity and in future, a different effective strategy for cell penetration may be advisable.

In the present assay, the control MA peptides 6C and 9C, which cover MA(51–65) and MA(81–95), respectively, showed significant anti-HIV activity. This is consistent with the previous studies, in which MA(41–55), MA(47–59) and MA(71–85) showed anti-HIV or dimerization inhibitory activity as discussed above.[16–18] These peptides have no R_8 sequence and thus cannot penetrate cell membranes. They exhibit inhibitory activity on the surface of cells, not intracellularly.

The structures of MA peptides 8L and 9L, dissolved in PBS buffer (2.7 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 9.59 mM Na_2HPO_4) at pH 7.4, were determined by CD spectroscopy (Fig. 3). When peptides form α-helical structures, minima can be observed at approximately 207 and 222 nm in their CD spectra. The amino acid residues covering fragments 8 and 9 corresponding to 8L and 9L are located in an α-helical region (helix 4) of the parent MA protein (Fig. 4), and peptides 8L and 9L were presumed to have an α-helical conformation.[26–28] However, the CD spectra shown in Figure 3, suggest that these peptides lack any characteristic secondary structure. This is because the 15-mer peptide derived from MA is not sufficiently long to form a secondary structure even though Gly, Cys and octa-Arg are attached to their C-terminus. Analysis of the CD spectra suggests MA fragment peptides need a longer sequence in order to form a secondary structure. The CD spectra of the control MA peptides 8C and 9C were not determined because the aqueous solubility of these peptides is inadequate.

Fluorescent imaging of live cells was used to evaluate the cell membrane permeability of the MA peptides 8L and 14L, which showed high and zero significant anti-HIV activity, respectively. The MA fragment 14 is a hybrid of the fragments 2 and 3, and the MA peptides 14L and 14C, which are based on the conjugation of the N-terminal chloroacetyl group of an R_8 peptide and iodoacetamide to the thiol group of the Cys residue, respectively (Supplementary data), are control peptides lacking significant anti-HIV activity (Tables 1 and 2). These peptides were labeled with 5(6)-carboxyfluorescein via a GABA linker at the N-terminus to produce 8F–L and 14F–L (Fig. 5A). The fluorophore-labeled peptides 8F–L and 14F–L were incubated with live cells of HeLa, A549 and CHO-K1, and the imaging was analyzed by a fluorescence microscope (Fig. 5B and C). A549 cells are human lung adenocarcinomic human alveolar basal epithelial cells.[29] Similar penetration of both peptides 8F–L and 14F–L into these cells was observed. Even peptides without significant anti-HIV activity can penetrate cell membranes. The penetration efficiency of both peptides into A549 was relatively high and into HeLa was low. In CHO-K1 the penetration efficiency of 8F–L is relatively low, but that of 14F–L is high. These imaging data confirm that the MA peptides with the R_8 sequence can penetrate cell membranes and suggest that MA peptides such as 8L and 9L should be able to inhibit HIV replication inside cells.

4. Conclusions

Several HIV-1 inhibitory fragment peptides were identified through the screening of an overlapping peptide library derived from the MA protein. Judging by the imaging experiments, peptides possessing the R_8 group can penetrate cell membranes and might exhibit their function intracellularly thus inhibiting HIV replication.

Two possible explanations for the inhibitory activity of these MA fragment peptides can be envisaged: (1) The fragment peptides might attack an MA protein and inhibit the assembly of MA proteins. (2) These peptides might attack a cellular protein and inhibit its interaction with MA. Further studies to elucidate detailed action mechanisms and identify the targets of these peptides will be performed in future. The technique of addition of the R_8 group to peptides enabled us to screen library peptides that function within cells. Thus, the design of an overlapping peptide library of fragment peptides derived from a parent protein with a cell membrane permeable signal is a useful and efficient strategy for finding potent cell-penetrating lead compounds.

In the present study, the MA peptides 8L and 9L were shown to inhibit HIV-1 replication with submicromolar to micromolar EC_{50} values in cells using the MT-4 assay (NL4-3 and NL(AD8) strains) and the p24 ELISA assay (JR-CSF strain). Our findings suggest that these peptides could serve as lead compounds for the discovery of novel anti-HIV agents. Amino acid residues covering fragments 8 and 9 corresponding to 8L and 9L are located in the exterior surface of MA, and in particular in the interface between two MA trimers (Fig. 4C).[26–28] The interaction of two MA trimers leads to the formation of an MA hexamer, which is the MA assembly with physiological significance. Thus, the region covering fragments 8 and 9 is critical to oligomerization of MA proteins. This suggests that MA peptides 8L and 9L might inhibit the MA oligomerization through competitive binding to the parent MA, and that more potent peptides or peptidomimetic HIV inhibitors could result from studies on the mechanism of action of these MA peptides and identification of the interaction sites. Taken together, some seeds for anti-HIV agents are inherent in MA proteins, including inhibitors of the interaction with PM such as the MA peptide 2C.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare. C.H. and T.T. were supported by JSPS Research Fellowships for Young Scientists. The authors thank Ms. M. Kawamata, National Institute of Infectious Diseases, for her assistance in the anti-HIV assay. We also thank Dr. Y. Maeda, Kumamoto University, for providing PM1/CCR5 cells, and Mr. S. Kumakura, Kureha Corporation, for providing SCH-D, respectively.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.055.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 27, Number 5, 2011

DOI: 10.1089/aid.2010.0191

Isolation of Potent Neutralizing Monoclonal Antibodies from an SIV-Infected Rhesus Macaque by Phage Display

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Abstract

The humoral immune response is a mechanism that potently suppresses or prevents viral infections. However, genetic diversity and resistance to antibody-mediated neutralization are serious obstacles in controlling HIV-1 infection. In this study, we isolated monoclonal antibodies from an SIV-infected macaque by using the phage display method to characterize antibodies in SIV infection. Variable regions of immunoglobulin genes were amplified by rhesus macaque-specific primers and inserted into the phagemid pComb3X, which produced the Fab fragment. Antibodies against SIV proteins were selected by biopanning using an SIV protein-coated 96-well plate. A total of 20 Fab clones obtained included 14 clones directed to gp41, four clones to gp120, and two clones to p27. The anti-gp120 Fab clones completely neutralized the homologous neutralization-sensitive SIVsmH635FC and the genetically divergent SIVmac316, and showed at least 50% inhibition against the neutralization-resistant strain, SIVsmE543-3. Competition ELISA revealed that these anti-gp120 Fab clones recognize the same epitope on gp120 including the V3 loop. Identification of antibodies with potent neutralizing activity will help to elucidate the mechanisms for inducing broadly neutralizing antibodies.

Introduction

THE HUMORAL IMMUNE RESPONSE is an important component of the adaptive immune response necessary to prevent viral infection and limit replication. Induction of these responses plays a key role in vaccine strategies against viral infection. Antibodies against human immunodeficiency virus type 1 (HIV-1), similar to those against other viruses, are capable of clearing HIV-1 virions.[1] Passive transfer of neutralizing antibodies (NAb) against HIV-1 to rhesus macaques provides sterilizing immunity against challenge with simian immunodeficiency virus (SIV)/HIV-1 chimeric viruses bearing the HIV-1 Env.[2–5] Although antibodies against a specific strain of HIV-1 can be highly potent, antibodies that neutralize a broad spectrum of HIV-1 strains are rarely induced in HIV-1-infected patients.[6,7] Since broad neutralization is critical for the development of a prophylactic vaccine against HIV-1, individuals who develop broadly NAbs are the subject of intense research.[8–12] In addition to the wide genetic diversity of HIV-1 limiting cross-neutralization, some strains of HIV-1 appear to be highly resistant to antibody-mediated neutralization. Many primary HIV-1 strains are resistant to neutralization, likely due to the structure of HIV-1 Env, which protects conserved regions from antibodies.[8,9,13] Consistent with the model of shielding of neutralizing epitopes, no broadly NAb has been obtained by immunization of mice or other animals with HIV-1 Env. Therefore, broadly neutralizing monoclonal antibodies (MAb) from HIV-1-infected patients are an essential but rare tool to understand the mechanism of neutralization against a broad spectrum of HIV-1 strains, including neutralization-resistant strains.

Macaques infected with SIV, which is genetically and biologically similar to HIV-1, are widely used as an animal model for HIV-1 infection.[14] The humoral immune response to SIV has been studied in the development of vaccine candidates and for exploration of antibodies that efficiently control viral infection. However, some SIV strains are known to be highly resistant to antibody neutralization, similar to the most resistant primary isolates of HIV-1.[15–17] The lack of MAbs that can neutralize these neutralization-resistant SIV strains is a major obstacle in the study of the mechanism of efficient neutralization using the SIV model.

Because conventional hybridoma technology is problematic in nonhuman primates, MAbs have been generated from rhesus macaques by the phage display method[18–21] or by transformation of B cells using rhesus Epstein–Barr virus-like virus.[22,23] In the present study, we used the phage display method to obtain MAbs against SIV antigens from an

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SIV-infected rhesus macaque with robust envelope-specific antibody responses. By panning with whole SIV antigen, we retrieved MAbs specific for SIV Env gp120, gp41, and Gag p27, and demonstrated the neutralization of a neutralization-resistant strain, SIVsmE543-3, by the gp120-specific MAbs.

Materials and Methods

Cells and viruses

PM1 cells[24] were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. TZM-bl,[25–28] GHOST(3) Hi-5,[29] and 293T[30] cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Lymphocytes from inguinal lymph nodes of SIVsmH635FC-infected rhesus macaque, H723,[31–33] were provided by Dr. Vanessa M. Hirsch. This animal died at 68 weeks postinfection with thrombus, bacterial endocarditis, and lymphadenopathy.[33] Peripheral blood samples were obtained from naive rhesus macaques, MM327, MM464, and MM449. These macaques were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University, Japan. Lymphocytes were isolated from blood samples by Ficoll gradient, and stored in liquid nitrogen until RNA preparation.

Infectious molecular clones SIVsmE543-3,[15] SIVsmH635FC,[32] SIVmac239,[34] SIVmac316,[35] and HIV-2_{GH123}[36] were transfected into 293T cells. After 2 days, the supernatants were filtered (0.45 μM) and stored at −80°C as virus stocks. The SIV antigen (Ag) used for panning was prepared by infection of PM1 cells with SIVsmE543-3. The culture supernatant was collected daily after 1 week postinfection and centrifuged at 12,000 rpm for 90 min at 4°C. After discarding the supernatant, the antigen pellet was resuspended in the remaining medium, treated with the same volume of PBS containing 1% Triton X-100, and stored at −80°C as SIV Ag.

Construction of Fab libraries

The Fab library was constructed using the pComb3X system according to instructions by Barbas *et al.*[37] The phagemid vector pComb3X was designed to express Fab or other proteins on the surface of phage with HA-tag and His-tag. Total RNA from lymphocytes was prepared using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). First-strand cDNAs were synthesized using oligo$(dT)_{20}$ primer and ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed by Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). The first round of PCR was performed to amplify immunoglobulin (Ig) heavy chain variable region (VH), and light chain κ and λ variable regions (Vκ and Vλ) from cDNA sample using primers shown in Table 1 using the following condition: 94°C for 30 s, followed by 30 cycles of 94°C for 15 s, 55°C for 15 s, and 68°C for 60 s. Ig heavy chain γ constant domain 1 (CH1) and Ig light chain κ and λ constant domains (Cκ and Cλ) were similarly amplified using phagemid pComb3X with rhesus Ig genes as templates. These template phagemids were constructed by inserting PCR products that were amplified from rhesus macaque cDNA using primers, VH1a-*Xho*I (5′-CAG GTG CAG CTC GAG

Table 1. Oligonucleotide Primers Used to Construct Fab Libraries

VH 5′ sence primers	
RhFabVH17-F	GCTGCCCAACCAGCCATGGCCCAGGTSCAGCTGGTGCAGTCYGG
RhFabVH2-F	GCTGCCCAACCAGCCATGGCCCAGGTGACCTTGAAGGAGTCTGG
RhFabVH35-F	GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGGTGSAGTCTGG
RhFabVH46-F	GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGCAGGAGTCRGG
VH 3′ reverse primers	
RhFabVHJ1-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCAGGGCGCC
RhFabVHJ2-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGATGGTGATTGGGGTGCC
RhFabVHJ36-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACSMYGASCCC
RhFabVHJ45-B	CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCAGGACTCC
Vκ 5′ sence primers	
RhSCK1-F	GGGCCCAGGCGGCCGAGCTCCAGATGWCCCAGTCTCC
RhSCK2-F	GGGCCCAGGCGGCCGAGCTCGTGATGAYCCAGACTCC
RhSCK23-F	GGGCCCAGGCGGCCGAGCTCGTRATGACKCAGTCTCC
RhSCK5-F	GGGCCCAGGCGGCCGAGCTCATACTCACACAGTCTGC
Vκ 3′ reverse primers	
RhCK5-B	GAAGACAGATGGTGCAGCCACAGC
Vλ 5′ sence primers	
RhSCLam131011	GGGCCCAGGCGGCCGAGCTCGDGCTGACWCAGCCACCCTC
RhSCLam2	GGGCCCAGGCGGCCGAGCTCGCCCYGACTCAGYCTCCCTCTGT
RhSCLam15	GGGCCCAGGCGGCCGAGCTCGTGCTGACSCAGCCKCCYTC
RhSCLam3a	GGGCCCAGGCGGCCGAGCTCGAGCTGACTCAGGAGCCTGCATTGTC
RhSCLam4	GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGTCGCCYTC
RhSCLam59	GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCRDCCTC
RhSCLam6	GGGCCCAGGCGGCCGAGCTCGTGTTCACTCAGCCCCATTC
RhSCLam78	GGGCCCAGGCGGCCGAGCTCGTRGTGACYCAGGAGCCMTC
Vλ 3′ reverse primers	
HCL5-B	CGAGGGGGCAGCCTTGGGCTGACC

CAG TCT GGG-3′) and CH-*Spe*I (5′-AGG TTT ACT AGT ACC ACC ACA TGT TTT TAT CTC-3′) for Ig heavy chain, VK1a-*Sac*I (5′-GAC ATC GAG CTC ACC CAG TCT CCA-3′) and CK-*Xba*I (5′-GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G-3′) for Ig light chain κ, and VL1-*Sac*I (5′-GGG CCC AGG CGG CCG AGC TCG TGC TGA CGC AGC CTC CCT C-3′) and CL2-*Xba*I (5′-GCG CCG TCT AGA CCT ATG AAC ATT CTG CAG G-3′) for Ig light chain λ. Sequence data of Ig clones were submitted to GenBank under accession numbers FJ795797–FJ795868. The phagemids pComb3X-327w4LK10-327w4HC12 and pComb3X-327w4LL8 were used for this amplification as templates (GenBank accession numbers FJ795816, FJ795838, and FJ795863) using the following primers: HIgGCH1-F (5′-GCC TCC ACC AAG GGC CCA TCG GTC-3′), dpseq (5′-AGA AGC GTA GTC CGG AAC GTC-3′), RhKC-F (5′-CGA GCT GTG GCT GCA CCA TCT GTC-3′), HLC-F (5′-GGT CAG CCC AAG GCT GCC CCC-3′), and Lead-B (5′-GGC CAT GGC TGG TTG GGC AGC-3′). In second-round PCR, the heavy and light chains were constructed from the purified VH and CH1, Vκ and Cκ, and Vλ and Cλ fragments by overlap extension PCR using primers LeadVH (5′-GCT GCC CAA CCA GCC ATG GCC-3′), dpseq, RSC-F (5′-GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC-3′), and Lead-B: 94°C for 30 s, followed by 15 cycles of 94°C for 15 s, 55°C for 15 s, and 68°C for 90 s. Third-round PCR generated final Fab products by overlap extension PCR of heavy and light chains using primers RSC-F and dp-EX (5′-GAG GAG GAG GAG GAG GAG AGA AGC GTA GTC CGG AAC GTC-3′): 94°C for 30 s, followed by 10 cycles of 94°C for 15 s, 55°C for 15 s, and 68°C for 3 min. All PCR products were purified using the QIAquick Gel Extraction Kit or QIAEX II Gel Extraction Kit (QIAGEN) after loading them on an agarose gel. The final Fab fragments were ligated with pComb3X after digestion with *Sfi*I. The ligation mix was used for transformation of XL1-Blue (Stratagene, La Jolla, CA) by electroporation. Transformed cultures were incubated in SB medium with 50 μg/ml carbenicillin, 10 μg/ml tetracycline, and 1.4 μg/ml kanamycin overnight at 37°C after adding VCSM13 helper phage (Stratagene). Library phage stock was obtained from the culture medium by PEG 8000/NaCl precipitation. Library size was determined by colony-forming units (CFU) after infection of XL1-Blue with a diluted phage sample.

Biopanning using SIV Ag

Panning was performed using SIV Ag. Briefly, a MaxiSoap 96-well plate (Thermo Fisher Scientific, Waltham, MA) was coated with 50 μl/well of SIV Ag, which was 5-fold diluted with phosphate-buffered saline (PBS), for 1 h at 37°C. The 5-fold dilution was used because the signal by enzyme-linked immunosorbent assay (ELISA) was the strongest at this dilution. Wells were washed twice with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with 5% skim milk (Wako Pure Chemical Industries, Osaka, Japan) in PBS (MPBS) for 1 h at 37°C. After discarding the blocking solution, 50 μl of phage library was added to each well, and the plate was incubated for 2 h at 37°C. After washing five times with PBS-T, bound phage were eluted with 50 μl 100 mM glycine (pH 2.2), and amplified for the next round of panning. A total of seven rounds of panning was performed.

To select Fab clones to SIV, phagemid DNA was transformed into TOP10F′ cells (Invitrogen). Bacterial colonies were cultured for 5 h at 37°C and Fab production was induced by adding isopropyl-ß-D-thiogalactopyranoside (IPTG, Wako Pure Chemical Industries) at a final concentration of 2 mM and shaking overnight at 37°C.

Large-scale Fab production and purification

A single colony was selected and cultured in 10 ml SB with 50 μg/ml carbenicillin at 30°C overnight. The bacterial culture was added to 200 ml to 1 liter SB medium with 20 mM $MgCl_2$ and 50 μg/ml carbenicillin and cultured for 8 h at 37°C. Fab production was induced by culturing overnight after adding IPTG at 1 mM. The bacterial pellet was resuspended in BugBuster Master Mix (Novagen, Madison, WI), and the soluble fraction was extracted according to the manufacturer's instruction. The clarified extract was loaded onto His GraviTrap (GE Healthcare, Buckinghamshire, UK) to purify histidine-tagged Fab. Purified Fab was concentrated and buffer exchanged to PBS by Vivaspin 6, 10 kDa MWCO (GE Healthcare).

ELISA assay to detect anti-SIV Fab

ELISA was performed to detect anti-SIV Fab. A MaxiSoap 96-well plate was coated with 50 μl/well of the 10-fold diluted SIV Ag for 1 h at 37°C. Wells were washed three times with PBS-T and blocked with MPBS for 1 h at 37°C or overnight at 4°C. Samples were added to each well at 50 μl/well, together with 50 μl of MPBS, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-T, 100 μl of anti-HA-peroxidase (1:1000 dilution; 3F10, Roche Molecular Biochemicals, Mannheim, Germany) was added to each well, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-T, 100 μl/well ABTS solution (Roche) was added, and the plate was incubated for 30 min at 37°C. The optical density (OD) at 405 nm was measured with reference OD_{490} using microplate reader (Model 550, Bio-Rad, Hercules, CA). The dilution of SIV Ag was determined from the result of ELISA using serially diluted SIV Ag. A sufficient signal was confirmed at the 40-fold dilution, and the 10-fold dilution was used for assays.

Con A SIV ELISA was performed to efficiently detect Fabs against gp120.[38] The plate was coated with PBS containing 50 ng/ml Con A (Sigma, St. Louis, MO) for 1 h at 37°C before adding SIV Ag to immobilize gp120, and processed similarly with SIV ELISA.

Competition ELISA

To determine the epitope specificity of Fab clones against gp120, a competition assay was performed using Con A ELISA. Ag-coated wells were incubated with 50 μl MPBS and 25 μl of serial dilutions of competitor, IgG-B404, for 1 h at 37°C. Subsaturating concentrations of Fab clones or murine MAbs, KK8, KK17, KK42, KK45, KK46, KK65, KK68,[39,40] and VM.18S, were added to each well at 25 μl/well, and the plate was incubated for 1 h at 37°C. After washing three times with PBS-T, 100 μl of anti-HA-peroxidase (1:1000) for Fab clones or antimurine IgG peroxidase (1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for murine MAbs was added to each well, and the plate was processed similarly with Con A ELISA.

Nucleic acid sequence analysis

For analysis of the Ig variable region, phagemid clones were sequenced using primers ompseq and pelseq.[37] Identical clones and defective clones were not used for further study. Complementarity-determining region 3 (CDR3) and the closest V gene allele were determined by comparison with human Ig genes using IMGT/V-QUEST in the International Immunogenetics Database (IMGT, http://imgt.cines.fr/).[41] Sequence data of Ig clones obtained were submitted to GenBank under accession numbers HM044964–HM045003.

Analysis of neutralizing antibody titers

A neutralization assay for kinetic analysis of antibody response in H723 was performed using GHOST(3) Hi-5 cells as previously described.[31] The highest dilution of plasma that resulted in a reduction in the number of GFP^+ cells by more than 90% is shown as the neutralization titer.

For screening of Fab clones, neutralizing ability was measured as the reduction in SIV^+ cells after infection to TZM-bl cells with SIVsmH635FC. Briefly, 100 μl of 10- and 30-fold diluted crude Fab samples in duplicate was incubated with 50 μl of 200 50% tissue culture infectious dose ($TCID_{50}$) of virus in a 96-well plate. After incubation for 1 h at 37°C, 100 μl of 1×10^5 TZM-bl cells/ml containing 18.75 μg/ml DEAE-dextran was added to each well. After 3 days, cells were washed with PBS, and fixed with PBS containing 2% formalin and 0.2% glutaraldehyde for 10 min at room temperature (RT). After washing twice, cells were stained with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl -β-D-galactopyranoside (X-Gal) in PBS containing 5 mM potassium hexacyanoferrate trihydrate, 5 mM potassium hexacyanoferrate, and 2 mM $MgCl_2$ for more than 1 h at 37°C. Fab clones that showed the reduction of stained cells were selected.

For the neutralization assay using purified Fab and plasma samples, the neutralizing ability was measured as the reduction in luciferase activity after infection to TZM-bl cells with various SIV strains, as previously described.[42] Briefly, 100 μl of serially diluted Fab samples in duplicate was incubated with 50 μl of 200 $TCID_{50}$ of virus in a 96-well plate for 1 h at 37°C, following addition of 100 μl of 1×10^5 cells/ml TZM-bl cells containing 37.5 μg/ml DEAE. Infected cultures were incubated for 2 days, but cultures infected with SIVsmH635FC were incubated for 3 days. After incubation, cells were lysed with 30 μl of cell lysing buffer (Promega, Madison, WI) for 15 min at RT, and 10 μl of cell lysate was transferred to a 96-well black solid plate (OptiPlates-96F, Perkin-Elmer, Boston, MA) for measurements of luminescence using a GloMax 96 Microplate Luminometer (Promega) and the luciferase assay system (Promega). The 50% and 90% inhibitory concentrations (IC_{90} and IC_{50}) were defined as the Fab concentration that caused a 50% and 90% reduction in luciferase activity compared to virus control wells after subtraction of background, respectively.

Western blot assay

Reactivity of plasma antibodies to SIV proteins was assessed by immunoblotting. SIV Ag was diluted with twice the volume of sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis on 10% gel. Proteins were transferred to a Hybond-P PDVF membrane (GE Healthcare). The membrane was blocked with MPBS containing 0.1% Tween 20 for 1 h at RT, washed four times with PBS-T, and air dried on a filter paper. SIV strips were made by cutting the membrane, and stored at 4°C. For immunodetection, the SIV strip was incubated overnight at RT with 2 ml plasma from H723 (1:5000 dilution with MPBS containing 0.1% Tween 20), washed twice with PBS-T, and incubated for 1 h at RT with 2 ml protein A/G, and alkaline phosphatase conjugated (1:10,000 dilution with 0.5% milk PBS containing 0.1% Tween 20; Thermo Fisher Scientific). After washing three times with PBS-T, 1 ml BCIP/NBT solution (KPL, Gaithersburg, MD) was added to develop color.

Reactivity of Fab clones to SIV was also assessed using SIV strips. SIV strips were incubated overnight at 4°C with 1.8 ml MPBS containing 0.1% Tween 20 and 200 μl crude bacterial supernatant or purified Fab, washed twice with PBS-T, and incubated for 1 h at RT with 2 ml anti-HA-peroxidase (1:500 dilution; Roche). After washing three times with PBS-T, 1 ml TMB solution (KPL) was added to develop color. Bands for viral proteins were identified using SIV-positive sera and Donkey anti-IgG (H + L)-peroxidase (1:20,000 dilution; 709-035-149, Jackson ImmunoResearch, West Grove, PA) and confirmed by commercial kit (SIV Blot; ZeptoMetrix Corp., Buffalo, NY).

Flow cytometric analysis

The ability of Fabs to bind virus-infected cells was analyzed by flow cytometric analysis. Briefly, PM1 cells were infected with SIVsmE543-3, SIVmac239, SIVmac316, or $HIV\text{-}2_{GH123}$ and cultures were kept for 3 weeks by adding PM1 cells. Infected and uninfected cells were washed with PBS and adjusted to 5×10^6 cells/ml. For cell surface staining, 50 μl cells in PBS containing 0.2% BSA were incubated with 10 μl of 20 ng/μl Fab for 40 min at RT. After washing with PBS containing 0.2% BSA, cells were incubated with 50 μl of anti-HA (1:200; Roche) for 20 min at RT, followed by incubation with 50 μl of anti-rat-FITC (1:500; Santa Cruz Biotechnology) for 20 min at RT. For both surface and intracellular staining, 50 μl of 5×10^6 cells/ml PM1 cells was fixed by IC Fixation Buffer (eBioscience, San Diego, CA) for 20 min at RT and washed with permeabilization buffer (eBioscience) twice before incubation with Fab. Cells were stained similarly to the surface staining process, but permeabilization buffer was used for washing and dilution of antibodies. The stained cells were analyzed by guava easyCyte 8HT (Millipore, Billerica, MA). Percentages of Fab^+ cells were determined by gating cells with high FITC intensity using unstained cells as a negative control. Data analysis was performed using FlowJo (TreeStar, San Carlos, CA).

Conversion of Fab B404 into a complete rhesus IgG

Rhesus macaque heavy chain Ig genes were amplified from H723 cDNA by RT-PCR using primers, SPH-F (5′-GAG CTA GCG CCG CCA CCA TGG ACT GGA CCT GGA-3′) and SPH-R (5′-CGA AGC TTG CAC CGG TGG CTG CTG CCA CCA AG-3′) for the leader region and HIgCH1-F (5′-GCC TCC ACC AAG GGC CCA TCG GTC-3′) and CH-R (5′-TTG TTT AAA CTA TCA TTT ACC CGG AGA CAC GGA GA-3′)

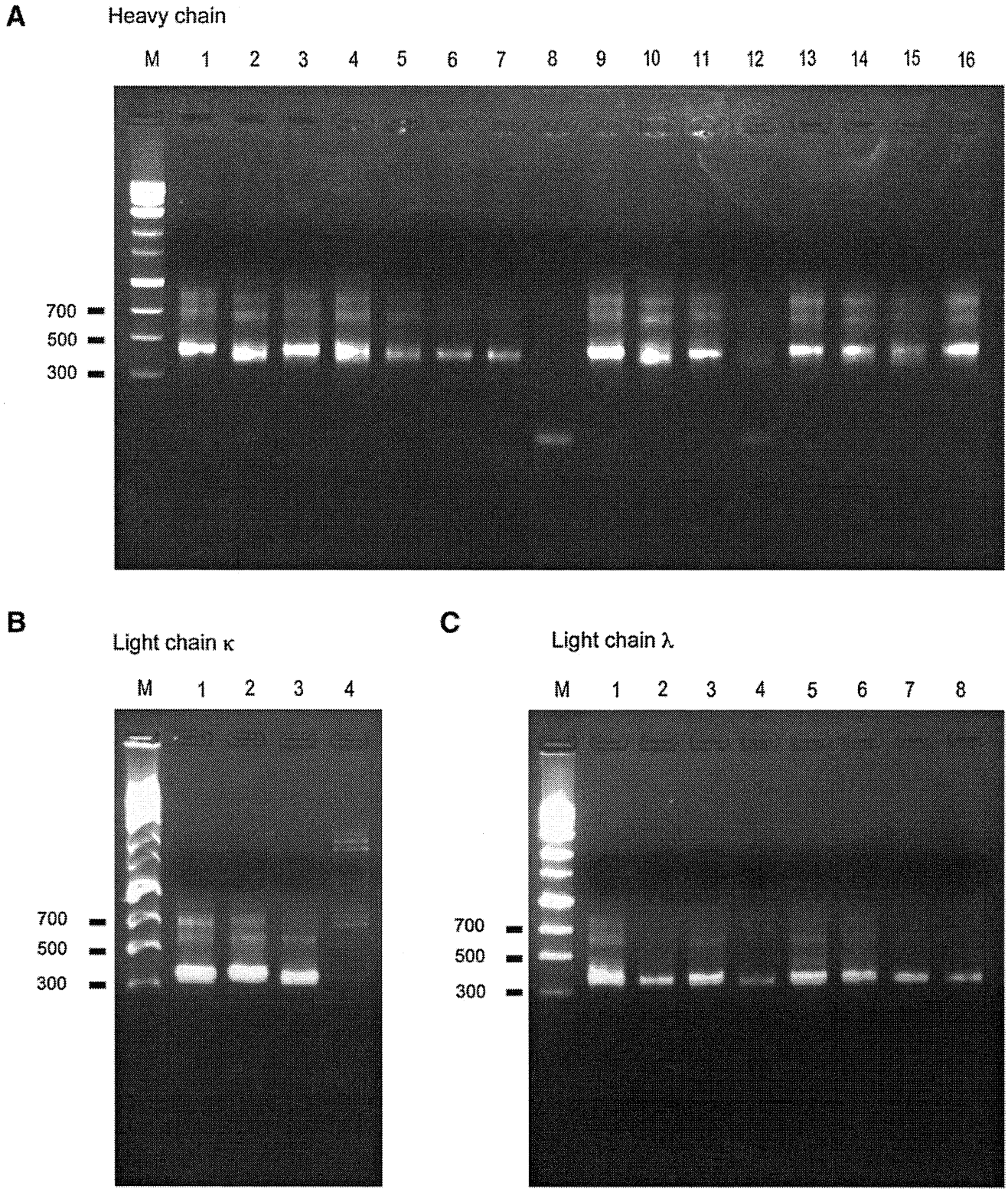


FIG. 1. Ig heavy chain and light chain κ and λ variable regions were successfully amplified by PCR using primers shown in Table 1. **(A)** The Ig heavy chain variable region was amplified using the following primer pairs: lane 1, RhFabVH17-F and RhFabVHJ1-B; 2, RhFabVH17-F and RhFabVHJ2-B; 3, RhFabVH17-F and RhFabVHJ36-B; 4, RhFabVH17-F and RhFabVHJ45-B; 5, RhFabVH2-F and RhFabVHJ1-B; 6, RhFabVH2-F and RhFabVHJ2-B; 7, RhFabVH2-F and RhFabVHJ36-B; 8, RhFabVH2-F and RhFabVHJ45-B; 9, RhFabVH35-F and RhFabVHJ1-B; 10, RhFabVH35-F and RhFabVHJ2-B; 11, RhFabVH35-F and RhFabVHJ36-B; 12, RhFabVH35-F and RhFabVHJ45-B; 13, RhFabVH46-F and RhFabVHJ1-B; 14, RhFabVH46-F and RhFabVHJ2-B; 15, RhFabVH46-F and RhFabVHJ36-B; and 16, RhFabVH46-F and RhFabVHJ45-B. **(B)** The Ig light chain κ variable region was amplified using RhCK5-B and the following primers: lane 1, RhSCK1-F; 2, RhSCK2-F; 3, RhSCK23-F; and 4, RhSCK5-F. (**C**) The Ig light chain λ variable region was amplified using HCL5-B and the following primers: lane 1, RhSCLam131011; 2, RhSCLam2; 3, RhSCLam15; 4, RhSCLam3a; 5, RhSCLam4; 6, RhSCLam59; 7, RhSCLam6; and 8, RhSCLam78. Template cDNA samples from four rhesus macaques were tested, and the representative result, which was obtained from macaque H723, is shown. M, molecular weight marker. The bands for 300 bp, 500 bp, and 700 bp are indicated on the left.

for the constant region. The leader and constant regions were inserted into pcDNA3.1(+) using restriction enzymes *Nhe*I and *Hin*dIII and *Apa*I and *Pme*I, respectively. The resultant plasmid, designated as pHCG, has an Ig heavy chain gene lacking the VH region. The VH region was amplified from Fab B404 using primers VH35-F (5′-GCC ACC GGT GCC CAC TCC GAG GTG CAG CTG GTG-3′) and VH-R (5′-CGA TGG GCC CTT GGT GGA G-3′) and inserted into pHCG after digestion with *Sgr*AI and *Apa*I. The B404 light chain gene, which was amplified using primers, Lam131011-F (5′-GDG CTG ACW CAG CCA CCC TC-3′) and CL2-*Xba*I, was combined with the light chain leader region, which was amplified using primers SPLa-F (5′-GAA AGC TTG CCG CCA CCA TGG CCT GGR CTC CWC-3′) and SPL131011-R (5′-GAG GGT GGC TGW GTC AGC HC-3′) by overlapping PCR using primers SPLa-F and CL2-*Xba*I. The PCR product containing the complete light chain gene was inserted into pcDNA3.1/Hyg(+) after digestion with *Hin*dIII and *Xba*I. The stable cell line expressing IgG-B404 was obtained by transfection of these plasmids into 293A cells and selection with 400 μg/ml G418 and 100 g/ml hygromycin. The culture supernatant was concentrated by Vivaflow 50, 30,000 MWCO (Sartorius Stedim Biotech, Goettingen, Germany), and IgG was purified by HiTrap rProtein A FF (GE Healthcare). Purified IgG was concentrated and buffer exchanged to PBS by Vivaspin 6, 10 kDa MWCO (GE Healthcare).

Results

Amplification of immunoglobulin V regions from macaque monkeys

Primers used to amplify variable regions of Ig genes were newly designed for rhesus macaques based on the primers for construction of a combinatorial library of human Ig genes,[37] germline sequences of rhesus Ig genes,[43–51] and the genome database of rhesus macaque[52] (Table 1). The Ig variable regions were amplified from lymphoid cells of SIVsmH635FC-infected macaque H723 and three uninfected macaques using these primers detailed in Table 1 (Fig. 1). Although most primers successfully amplified the Ig variable regions, some primers did not work well. For example, the reverse primer for the heavy chain, RhFabVHJ45-B, was not effective in amplifying a product in combination with the RhFabVH2-F and RhFabVH35-F primers (Fig. 1A, lanes 8 and 12). In addition, the sense primer for light chain κ, RhSCK5-F, was not effective (Fig. 1B, lane 4), even though this primer was designed based on a rhesus genome sequence that was similar to germline sequence of human IGKV5. Nevertheless, the

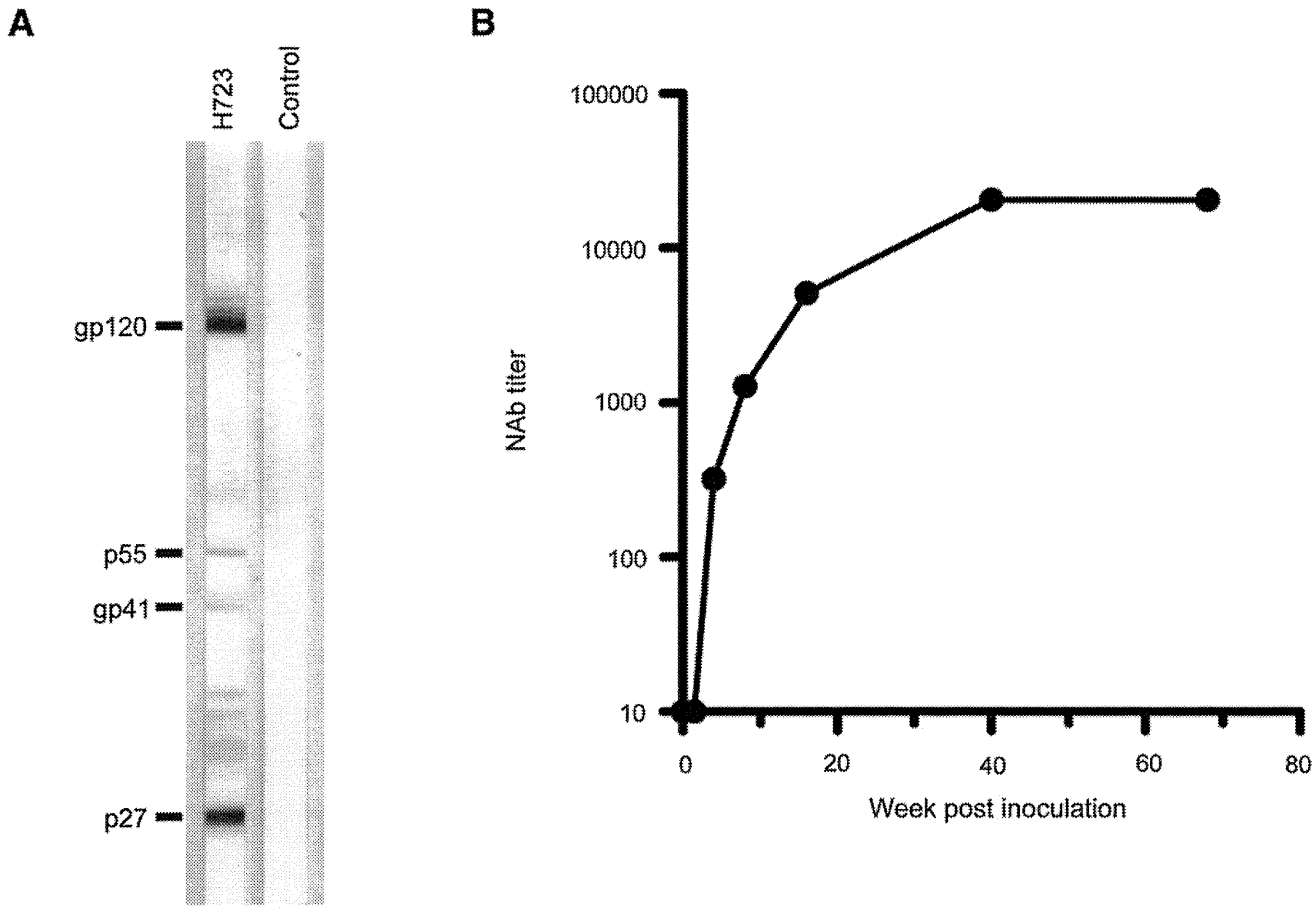


FIG. 2. Antibody response in SIVsmH635FC-infected macaque H723. **(A)** Western blot analysis showed that antibodies in plasma from H723 at death recognized Env gp120 and gp41 and Gag p55 and p27. **(B)** H723 maintained a high NAb titer during the course of infection. Neutralizing titer was determined by 90% inhibition of infection to GHOST(3) Hi-5 cells with SIVsmH635FC.

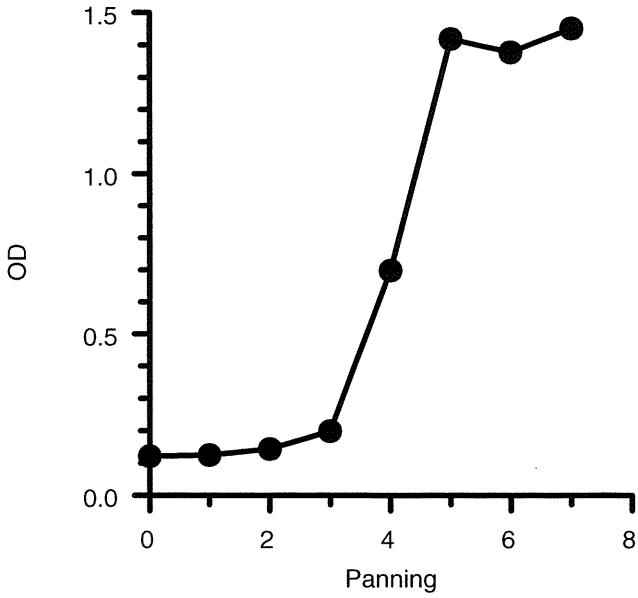


FIG. 3. Reactivity of phage to SIV Ag increased from the fourth round of panning. Phage samples that were eluted after panning were examined by ELISA for their ability to bind SIV proteins.

majority of these primer pairs worked with efficiency sufficient for the construction of the Ig library.

Construction of Fab library from SIVsmH635FC-infected macaque H723

The Fab library was constructed using the pComb3X system [37] from RNA extracted from lymph nodes of rhesus macaque H723, which was infected with a derivative of SIVsmE543-3, SIVsmH635FC.[31,32] This animal was used as a source of RNA since previous studies demonstrated that it mounted a vigorous antibody response.[31] Plasma collected at the time of death from H723 contained abundant antibodies against viral structural proteins Env and Gag (Fig. 2A). The NAb titer of plasma from this macaque sharply increased in acute infection and remained high until death (Fig. 2B), making this an attractive source of Ig genes for this study. The Ig variable regions, VH, Vκ, and Vλ, which were amplified from a lymph node of H723 (Fig. 1), were joined to conserved domains, CH1, Cκ, and Cλ, by overlap extension PCR, respectively. The final Fab gene fragment, which was generated by overlap extension PCR of heavy and light chains, was inserted into pComb3X after digestion with *Sfi*I. The resultant ligation mix was transformed into XL1-Blue *Escherichia coli* cells, and the phage library was prepared by adding helper phage. The size of the Ig library from macaque H723, estimated by the ability to transform XL1-blue, was 2.1×10^8 CFU.

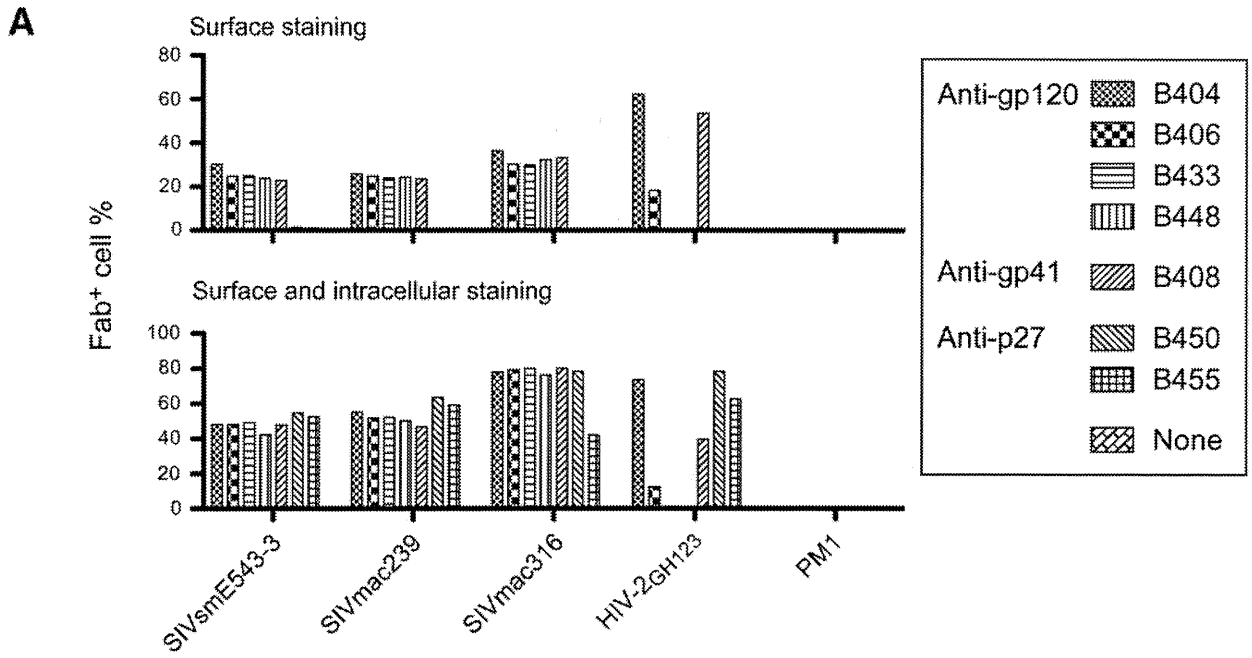
Selection of SIV-specific Fab clones by panning

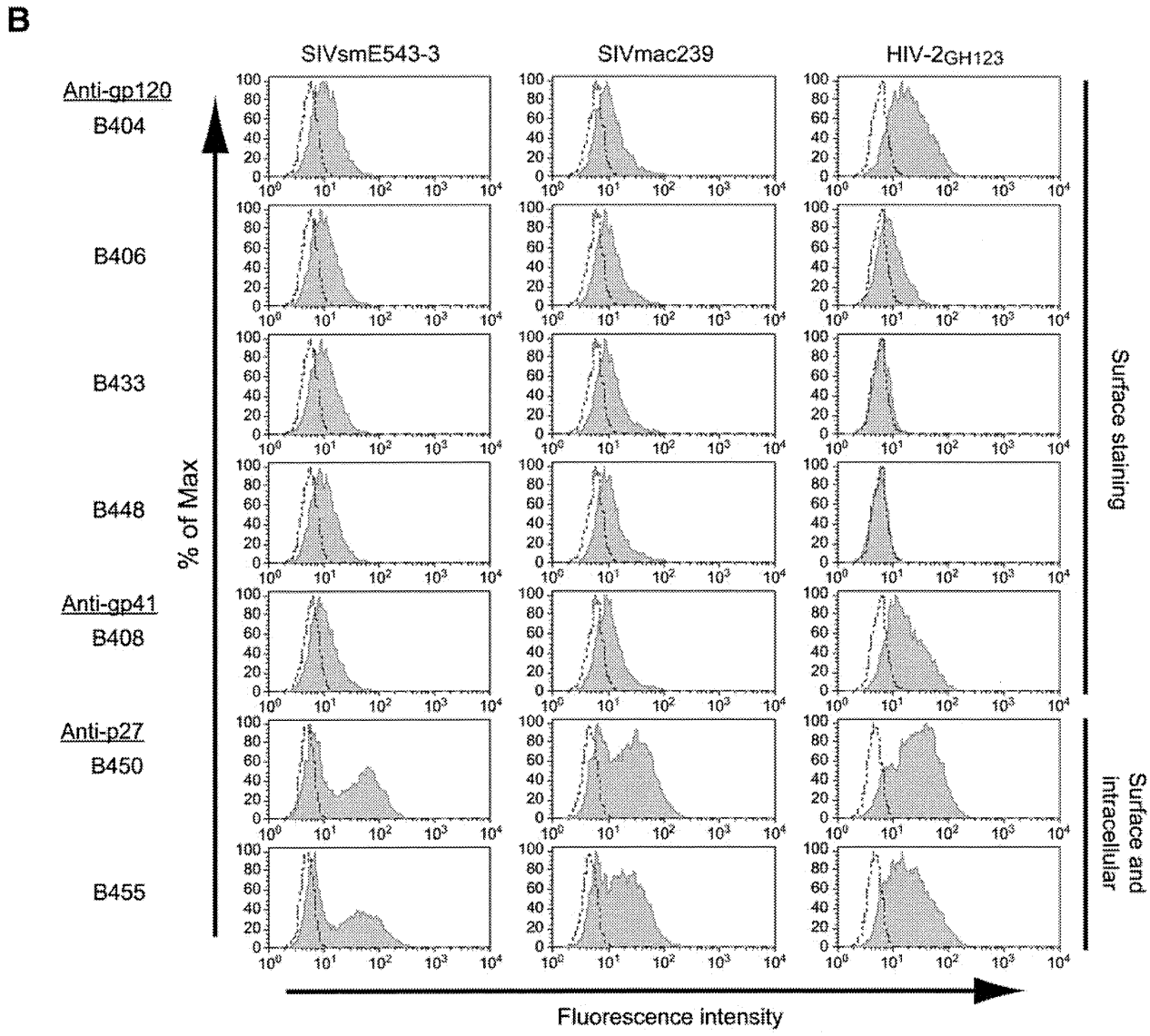
Biopanning was performed on a whole, Triton X-100-disrupted SIVsmE543-3 Ag-coated 96-well plate. The ability of phage to bind SIV Ag increased from the fourth round of panning, indicating successful selection of SIV-specific Fab (Fig. 3). Phagemid DNA was prepared from the fourth and fifth round of panning, and transformed into TOP10F′ *E. coli* cells to select clones that produce SIV-specific Fabs. Colonies were screened for reactivity of bacterial supernatants to SIV Ag using ELISA. SIV-specific Fab clones were sequenced, and a total of 20 independent clones were obtained (Table 2). Sequence analysis revealed that Fab clones expressed VH alleles, IGHV3 and IGHV4; Vκ alleles, IGKV1 and IGKV2; and Vλ

TABLE 2. CHARACTERISTICS OF FAB CLONES FROM AN SIV-INFECTED RHESUS MACAQUE

Clone	*VH allele*	*VH CDR3*	*Vκ or Vλ allele*	*Vκ or Vλ CDR3*	*WB*[a]	*NA*[b]
B404	IGHV3-h*01(P)[c]	TTGLQISEWFSTDGDEYFEF	IGLV3-25*02	QSSSGYHWV	gp120	>90%
B406	IGHV3-h*02(P)	VSGLQVSEWFSTDGDEYFEF	IGLV6-57*01	QSVDGTYNRL	gp120	>90%
B433	IGHV3-h*01(P)	SRGADFWSGSDRYFDF	IGLV3-25*02	HSVDSSAHHWV	gp120	>90%
B448	IGHV3-h*01(P)	TTGLQISEWFSADGDEFFEF	IGLV6-57*01	QSIDGYNRL	gp120	>90%
B402	IGHV4-4*07	IKQSYGRTV	IGKV2-40*01	MQGLDFPFT	gp41	—[d]
B405	IGHV4-28*01	ARRGGGPRARWFDV	IGKV2-40*01	MQALGFPPT	gp41	—
B407	IGHV4-4*07	ARRGVLRTSRIFDF	IGKV2-40*01	MQALGFPPT	gp41	—
B408	IGHV4-4*02	IKQSYGRTI	IGKV2-40*01	MQALQFPFT	gp41	—
B416	IGHV4-28*01	ARRGVYAGSRVFDF	IGKV2-40*01	MQAREFPPT	gp41	—
B417	IGHV4-4*02	VRRGVSAPAGTMLYFDL	IGKV2-40*01	MQGIESPPT	gp41	—
B418	IGHV4-4*07	IKQSYGRTI	IGKV2-40*01	MQGLDFPFT	gp41	—
B431	IGHV4-30-2*01	ARRGSYCSGNQCSRIFDS	IGKV2-40*01	MQALGFPPT	gp41	—
B434	IGHV4-4*02	VRRGVSAPAGTMLYFDL	IGKV2-40*01	MQGIESPPT	gp41	—
B438	IGHV4-28*05	ARRGVYAGSRVFDF	IGKV2-40*01	MQSLEFPPT	gp41	—
B442	IGHV4-28*01	ARRGVYAGSRVFDF	IGKV2-40*01	MQALGFPPT	gp41	—
B444	IGHV4-39*07	ARRGSICSGNQCSRIFDY	IGKV2-40*01	MQALGFPPT	gp41	—
B503	IGHV4-4*07	IKQSYGRTV	IGKV2-40*01	MQGLDFPFT	gp41	—
B505	IGHV4-b*01	ARRGVIGTSRIFDF	IGKV2-40*01	LQGLGFPPT	gp41	—
B450	IGHV4-39*07	ARQGPAAGVDS	IGKV1-17*01	LQHYSYPLT	p27	—
B455	IGHV4-4*07	ASHNFWSGPDY	IGLV4-69*01	QTWDTGIVL	p27	—

[a]Western blotting analysis was performed to determine the target viral protein.
[b]Neutralization assay was performed by infection of TZM-bl cells with SIVsmH635FC. Neutralization was shown by % inhibition of infection.
[c](P) pseudogene.
[d]—, < 50% inhibition.


A
Surface staining
Surface and intracellular staining
Fab+ cell %
Anti-gp120
B404
B406
B433
B448
Anti-gp41
B408
Anti-p27
B450
B455
None
SIVsmE543-3
SIVmac239
SIVmac316
HIV-2GH123
PM1


B
SIVsmE543-3
SIVmac239
HIV-2GH123
Anti-gp120
B404
B406
B433
B448
Anti-gp41
B408
Anti-p27
B450
B455
% of Max
Fluorescence intensity
Surface staining
Surface and intracellular

alleles, IGLV3, IGLV4, and IGLV6. Fourteen clones with the IGKV2-40*01 allele showed homologous CDR3 sequences, suggesting that these clones had the same origin.

Western blot assay was performed using crude bacterial supernatants to determine the target protein recognized by the each of these Fab clones (Table 2). Four Fab clones, B404, B406, B433, and B448, recognized Env gp120. Two clones, B450 and B455, recognized Gag p27. The other 14 clones, which had the predominant IGKV sequence, recognized Env gp41. The four anti-gp120 Fab clones were closely related to one another, but two anti-p27 Fab clones had distinct origins (Table 2). Identification of Fab clones against multiple proteins with multiple V gene alleles suggests that the library from SIVsmH635FC-infected macaque, H723, contains a wide variety of Fab genes against SIV.

Reactivity of Fabs to SIV or HIV-2-infected cells

Four anti-gp120 Fabs (B404, B406, B433, and B448), one anti-gp41 Fab (B408), and two anti-p27 Fabs (B450 and B455) were affinity purified and examined for their ability to bind virus-infected cells. Anti-gp120 and anti-gp41 Fabs efficiently bound cells infected with SIVsmE543-3, SIVmac239, and SIVmac316. However, reactivity to HIV-2_{GH123} differed among the Fab clones (Fig. 4A). B404 and B408 showed significant reactivity to HIV-2_{GH123}, whereas very weak to no reactivity was observed for B406, B433, and B448 (Fig. 4A and B). Anti-p27 Fab^+ cells were observed only after permeabilization, consistent with the cytoplasmic localization of the Gag protein (Fig. 4A). These Fabs cross-react with all the SIV strains and HIV-2_{GH123}, though the fluorescence intensity of B455 was low against HIV-2_{GH123} (Fig. 4B). Results showed that these Fabs efficiently bound diverse strains of SIVsm/mac, and some of them were cross-reactive with HIV-2.

Neutralizing activity of anti-gp120 Fabs against various SIV strains

The neutralizing activity of the Fab clones was tested using a neutralization assay against SIVsmH635FC (Table 2). All the crude bacterial supernatants from anti-gp120 Fab clones had high neutralizing activity, though anti-gp41 and anti-Gag Fab clones did not show any inhibitory effect. To analyze the spectrum and potency of neutralizing activity, we examined the capacity of purified Fabs to neutralize other SIV strains, SIVsmE543-3, SIVmac239, and SIVmac316, and HIV-2_{GH123} (Fig. 5). Consistent with the results from crude bacterial supernatants, purified anti-gp120 Fabs, B404, B406, B433, and B448, efficiently neutralized SIVsmH635FC, whereas the anti-gp41 Fab, B408, had no effect. Neutralization of SIVsmH635FC was achieved at a concentration of 16–80 ng/ml for IC_{90} and at 3.2–16 ng/ml for IC_{50} (Fig. 5A).

SIVsmE543-3, which is genetically close to SIVsm H635FC but considerably more resistant to antibody neutralization, was also neutralized by these anti-gp120 Fabs (Fig. 5B). The maximum inhibition of SIVsmE543-3 ranged from 60% to 80%, indicating that its neutralizing activity was moderate compared with that of SIVsmH635FC. However, IC_{50} against SIVsmE543-3 was 3.2 ng/ml, which was the same level as that of SIVsmH635FC. Furthermore, the broad spectrum of these anti-gp120 Fabs was shown by neutralization of the genetically heterologous, neutralization-sensitive SIV strain, SIVmac316 (Fig. 5D). Neutralization of SIVmac316 was similar to that of SIVsmH635FC. The low IC_{50} value against various SIV strains (3.2–16 ng/ml) demonstrated the potency of these Fabs since broad-spectrum anti-HIV-1 NAbs did not neutralize most of the primary HIV-1 strains at less than 10 ng/ml.[7]

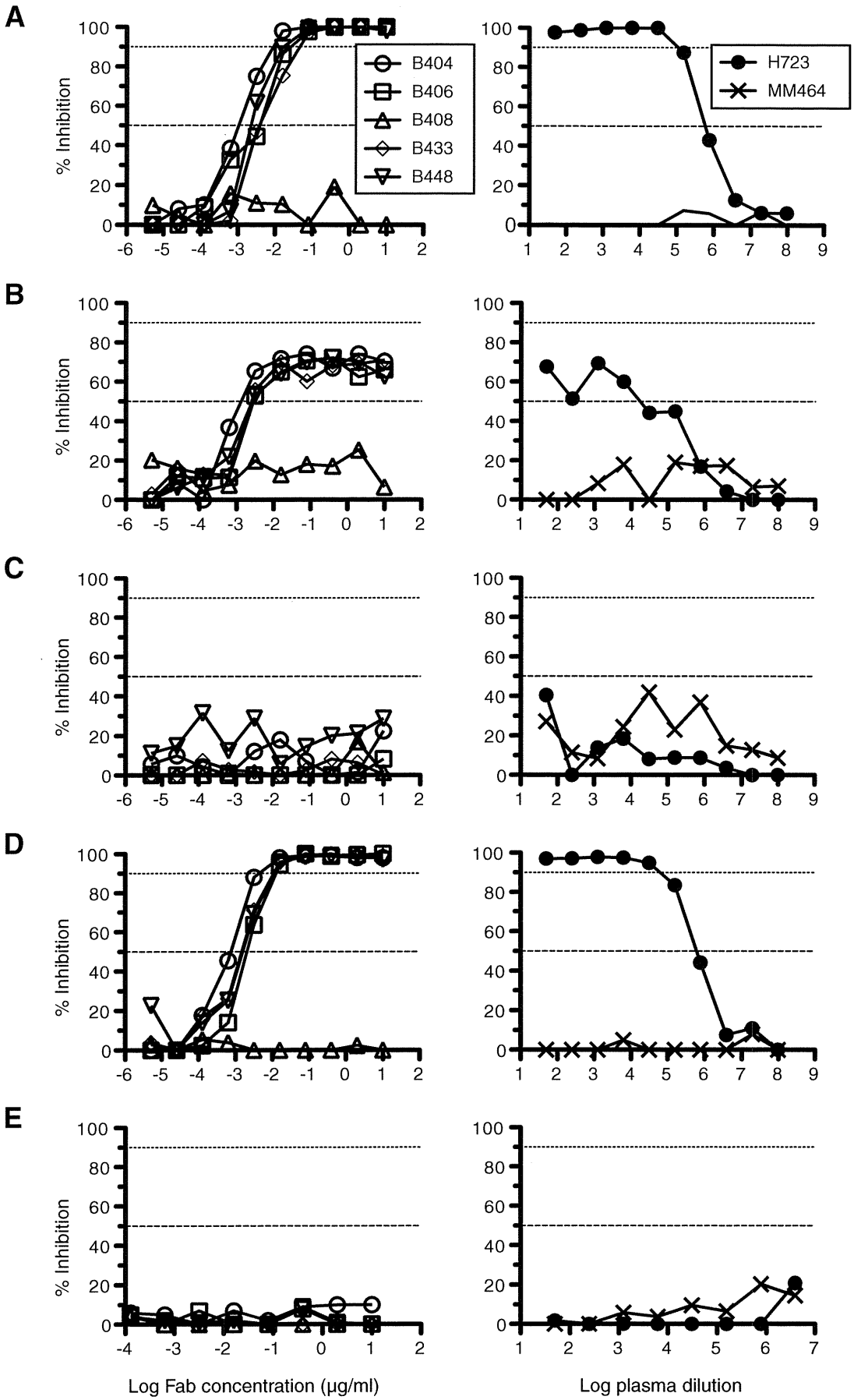
SIVmac239, another heterologous strain, and HIV-2_{GH123} were not neutralized by any Fab (Fig. 5C and E). HIV-2_{GH123} was not neutralized, perhaps because of its low cross-reactivity, though B404 bound HIV-2_{GH123} (Fig. 4). Unsuccessful neutralization of SIVmac239 may be related to the Env structure, which is highly resistant to antibody neutralization,[16,17] as well as the antigenic difference between SIVmac239 and SIVsmH635FC.

The neutralization pattern of these anti-gp120 Fabs was similar to that of the plasma sample of the macaque from which the library was constructed (Fig. 5, right panels). The neutralizing activity of H723 plasma was markedly high against SIVsmH635FC and SIVmac316, moderate against SIVsmH543, and extremely low against SIVmac239 and HIV-2_{GH123}. This similarity suggests that anti-gp120 Fabs, B404, B406, B433, and B448, may be representative NAbs in the host macaque.

Anti-gp120 Fab clones share the same epitope on gp120

To identify the epitope recognized by anti-gp120 Fab clones, we performed competition ELISA using IgG-B404, which was converted from Fab B404 to a complete rhesus IgG. The binding and neutralizing ability of IgG-404 was shown to be similar to Fab B404 by ELISA and neutralizing assay (data not shown). In the first experiment, the binding of anti-gp120 Fabs, B404, B406, B433, and B448, was examined in competition with IgG-B404 to determine whether these Fabs recognize the same epitope. As shown in Fig. 6A, all the anti-gp120 Fabs were inhibited with similar kinetics by IgG-B404. This competition suggests that these Fabs recognize the same epitope, or that there is an overlap in their epitopes. In the second experiment, murine MAbs were examined for their binding ability in competition with IgG-B404 to identify the epitope of B404. IgG-B404 competed with three murine MAbs, KK42,

FIG. 4. Ability of Fabs to bind virus-infected cells. Uninfected PM1 cells and PM1 cells that were infected with SIVsmE543-3, SIVmac239, SIVmac316, and HIV-2_{GH123} were incubated with Fab, B404, B406, B433, B448, B408, B450, and B455, and the reactivity of Fab was analyzed by flow cytometry. **(A)** Percentages of cell-surface Fab^+ cells without permeabilization (upper) and Fab^+ cells after staining both surface and intracellular staining by permeabilization (lower) are shown. **(B)** Flow cytometry profiles of PM1 cells infected with SIVsmE543-3, SIVmac239, and HIV-2_{GH123} are shown as representative samples. The results of surface staining were shown in Fab clones against gp120 and gp41, and those of surface and intracellular staining were shown in Anti-p27 Fab, as indicated on the right. The tinted histogram represents cells stained by the Fab indicated on the left. The dotted line shows unstained control.


A
B
C
D
E
% Inhibition
B404
B406
B408
B433
B448
H723
MM464
Log Fab concentration (μg/ml)
Log plasma dilution

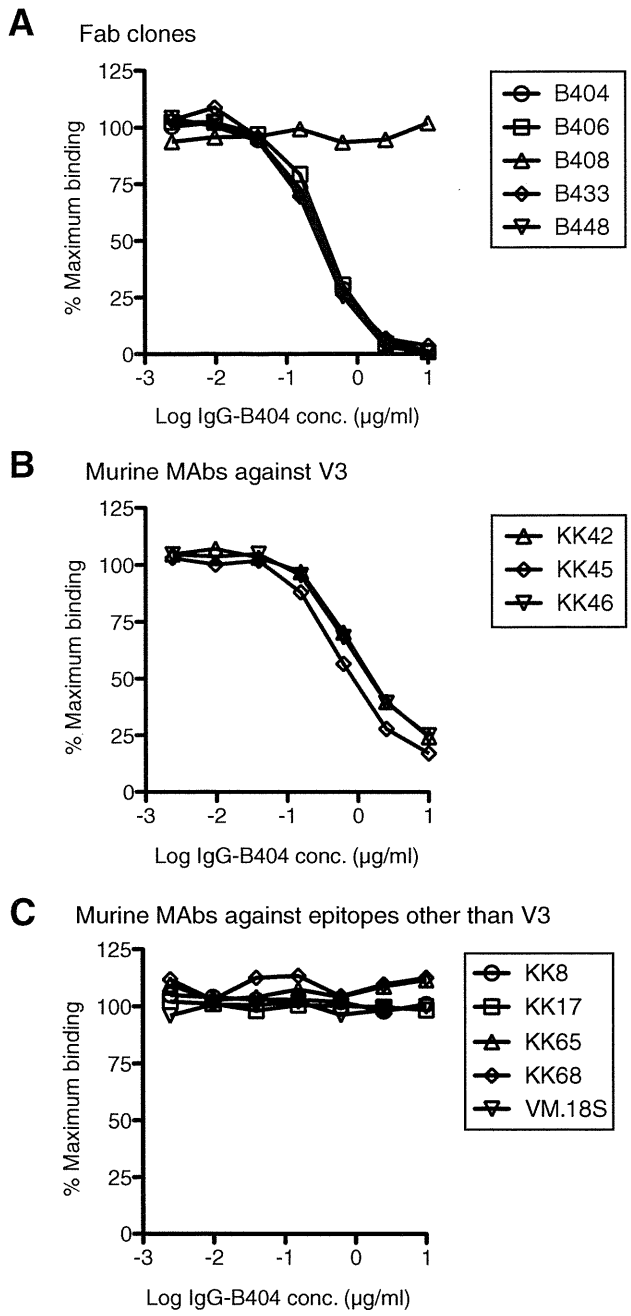


FIG. 6. Identification of epitope specificity of anti-gp120 Fab clones by competition ELISA. **(A)** IgG-B404 inhibited the binding of anti-gp120 Fabs, B404, B406, B433, and B448, but did not inhibit anti-gp41 Fab B406. **(B)** IgG-B404 competed with the binding of murine MAbs, KK42, KK45, and KK46, which recognize a linear epitope in the V3 loop of gp120. **(C)** IgG-B404 did not compete with murine MAbs, KK8 (V1/V2), KK17 (AA8-303), KK65 (V1), KK68 (C1), and VM.18S (unknown).

KK45 and KK46, which all recognize a linear epitope in the V3 loop of gp120 (Fig. 6B). In contrast, IgG-B404 did not compete with murine MAbs that target other epitopes (Fig. 6C). The results suggest that anti-gp120 Fabs, B404, B406, B433, and B448, recognize an epitope containing the V3 loop of gp120.

Discussion

We obtained a panel of MAbs against SIV from an SIV-infected rhesus macaque by using the phage display method. The use of a combinatorial library displayed on the phage surface is an efficient, fast, and well-established strategy to generate MAbs from infected or vaccinated donors.[37] The genomic structure of macaque Ig genes closely resembles that of human and germline sequences of macaque V, D, and J segments and shows high identity with those of humans.[43–51] Therefore, oligonucleotide primers for human Ig variable regions were used to amplify macaque variable regions. In this study, we modified primers for human Ig genes to improve their specificity for rhesus macaques. The successful selection of 20 monoclonal Fabs with multiple targets and origins, one of which is close to the human IGHV pseudogene, suggests that rhesus-specific primers were effective in amplifying sufficiently diverse Ig genes to select antibodies from rhesus macaque. Although identification of unknown germline sequences of rhesus macaque Ig genes is required for further improvement of the amplification system specific to rhesus Ig genes, the primers used in this study would clearly be valuable for constructing libraries to generate new MAbs from rhesus macaques.

Although we generated a diversity of Fab clones to SIV envelope and Gag proteins, Fab clones against gp41 comprised the majority. In addition, anti-gp41 Fab B402 and B405 were repeatedly obtained during the screening. One possible reason for the dominance of anti-gp41 Fab could be a bias in the panning procedure toward Fabs that bound this particular antigen. Apparently, the amount of gp120 was low in the absence of Con A,[38] consistent with the very low signal of anti-gp120 Fabs in ELISA. The signals of two anti-p27 Fabs in ELISA were also low compared with anti-gp41 Fabs, suggesting inefficient binding of p27 on the well. The repeated amplification of phage during panning, which excludes minor populations, might result in the dominance of anti-gp41 Fab, which was advantageous for binding to Ag coated on the plate. Selection of Fabs against particular epitopes may also result from the biased panning. Despite the biased selection, identification of Fabs against Env gp120, gp41, and Gag p27 in this study suggests that panning partially reflects antibody response *in vivo* because these viral proteins are major targets for antibodies in macaque H723 (Fig. 2).

Anti-gp120 Fabs, B404, B406, B433, and B448, showed neutralizing activity against three of the four SIV strains used in this study. Of these SIV strains, SIVsmH543-3 was shown to

FIG. 5. Anti-gp120 Fabs, B404, B406, B433, and B448, neutralized various SIV strains. Neutralizing activity against SIVsmH635 **(A)**, SIVsmE543-3 **(B)**, SIVmac239 **(C)**, SIVmac316 **(D)**, and HIV-2_{GH123} **(E)** was measured using Fabs (left panels) and plasma samples (right panels). Titration curves of percent inhibition of virus infectivity by anti-gp120 Fab clones, B404 (circles), B406 (squares), B433 (diamonds), and B448 (inverted triangles), are shown with anti-gp41 Fab B408 (triangles) as a negative control. A plasma sample from SIVsmH635-infected macaque H723 (black circles), from which the phage library was constructed, is compared with that from normal macaque MM464 (crosses). The 50% and 90% inhibitory doses are shown by the dotted line.

be resistant to antibody neutralization through analysis using sera that broadly neutralize genetically diverse SIV strains.[15] Fab B404, B406, B433, and B448 are the first MAbs that can neutralize SIVsmH543-3. The successful selection of these NAbs against neutralization-resistant SIV may be due to the robust antibody response in H723 from which the Fab library was constructed. H723 was inoculated with SIVsmH635FC, a derivative of SIVsmH543-3. The genomes of SIVsmH543-3 and SIVsmH635FC differ by only 15 nucleotides, but their sensitivities to neutralization are significantly different.[31,32] Inoculation of macaques with neutralization-sensitive SIVsmH635FC resulted in the emergence of revertants to SIVsmH543-3 and variants with diverse V1/V2 and V4 regions.[31] Antibody response to these mutant viruses is consistent with the identification of NAbs against SIVsmH543-3 in H723, though we did not analyze the neutralizing activity of plasma samples from H723 against these mutant viruses.

The relationship between SIVmac239 and SIVmac316 is similar to the relationship between SIVsmH543-3 and SIVsmH635FC in terms of genetic homology and sensitivity to neutralization. SIVmac239 is highly resistant to neutralization by polyclonal antisera or MAbs, though SIVmac316, which is genetically close to SIVmac239, is highly sensitive to neutralization.[16,17] Anti-gp120 Fabs, B404, B406, B433, and B448, showed efficient neutralization against SIVmac316, but no neutralization was observed against SIVmac239. Neutralization of SIVmac316 suggests that B404, B406, B433, and B448 have a broad neutralizing activity against diverse SIV strains because the similarity of the Env amino acid sequence is only 83.1% between SIVsmH635 and SIVmac316. Recognition of a conformational epitope by these anti-gp120 Fabs may make it possible to neutralize various SIV strains. Although the anti-gp120 Fabs were shown to recognize an epitipe including the V3 loop, amino acid sequences in the V3 loop was significantly different among SIV strains and HIV-2, to which these Fabs bound. The panning using antigen by Triton X-100 treatment, which did not cause the destruction of the protein secondary structure, may prompt an efficient selection of Fabs against conformational epitopes. Unsuccessful neutralization of SIVmac239 by these Fabs, despite their ability to bind SIVmac239, simply confirms that this virus is resistant to antibody neutralization. The mechanism of resistance may be similar to that observed in primary HIV-1 strains[8,9,13] and the Env structure would be important for resistance.[16,17]

The present study demonstrates that the phage display method is a powerful tool to obtain MAbs from rhesus macaques. This approach will help to identify antigens and epitopes recognized by the immune response during SIV infection. The use of the SIV virion as an antigen and monoclonal antibodies to capture viral protein will improve the efficiency of obtaining potent neutralizing antibodies. Fab clones with neutralizing activity will be useful to analyze the mechanism of broad neutralization using the SIV macaque model.

Acknowledgments

We thank Dr. Masafumi Takiguchi and his laboratory members for helpful discussion and support for the experiments. We thank Dr. Shinya Suzu for helpful advice about the expression system. The phagemid vector pComb3X was kindly provided by the Scripps Research Institute. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: PM1 from Dr. Marvin Reitz, GHOST(3) Hi-5 from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman, and TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc., KK8, KK17, KK42, KK45, KK46, KK65, and KK68 from Dr. Karen Kent and Miss Caroline Powell, and VM.18S from NIAID, DAIDS. We also thank Dr. Vanessa M. Hirsch for valuable advice. This work was supported in part by the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sport, Science, and Technology, Japan.

Author Disclosure Statement

No competing financial interests exist.

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