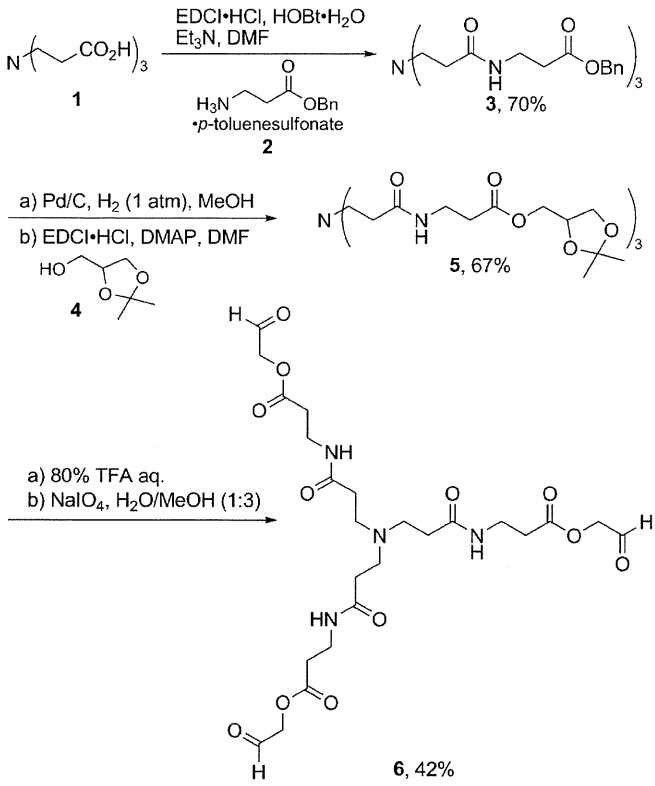
Scheme 1. Synthesis of the Equivalently Branched Template 6



antibody (2C2; 1:2000 dilution) (*20*) and anti- mouse IgG (H+L)-HRP (Millipore, MA). The band intensity of p24 was calculated with post/pre-immunized samples by using *ImageJ* image analyzing software.

RESULTS AND DISCUSSION

The N-region of gp41 is known to be an aggregation site involving a trimeric coiled-coil conformation. In design of an N36-derived peptide (N36RE), the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase the solubility in buffer solution (Figure 1B). In order to form a triple helix corresponding precisely to the gp41 prefusion form, we designed the novel C3-symmetric template depicted in Figure 1C. This designed template linker has three branches of equal length and possesses the hydrophilic structure and ligation site for coupling with N36RE. The template was synthesized from the commercially available 3-[bis(2-carboxyethyl)amino]propanoic acid **1** as shown in Scheme 1. Coupling of **1** with β-alanine benzyl ester **2** gave the corresponding triamide **3** in 77% yield. Cleavage of three benzyl esters by hydrogenation and coupling with solketal **4** produced the corresponding triester **5**. Deprotection of the acetonides with aqueous 80% TFA followed by oxidative cleavage of diol group led to the desired template **6**. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected N36RE (N36REGC) with a three-armed aldehyde scaffold producing triN36e (Figure 1D). Thiazolidine ligation is a peptide segment coupling strategy which does not require side chain protecting groups (*22*−*26*). The reaction consists of three steps: (i) aldehyde introduction, in which a masked glycolaldehyde ester is linked to the carboxyl terminus of an unprotected peptide by reverse proteolysis; (ii) ring formation, in which the unmasked aldehyde reacts at acidic pH with the α-amino group of an N-terminal cysteine residue of the second unprotected peptide forming a thiazolidine ring; and (iii) rearrangement at higher pH in which O-acyl ester linkage is converted to an N-acyl amide linkage forming a peptide bond with a pseudoproline structure (Figure 2).

Circular dichroism (CD) spectra of triN36e and N36RE, which is a monomer form without N-terminal Cys-Gly residues, are shown in Figure 3A. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, suitable for measurement of CD spectra of membrane proteins (*27, 28*). Both spectra display double minima at 208 and 222 nm and showed high molar ellipticity as absolute values (Table 1). The results indicate that these peptides form a highly structured α-helix and that the helical content of the trimer triN36e is higher than that of the monomer N36RE. Furthermore, to assess the interaction of triN36e with C34, CD spectra of the peptide mixture with C34-derived peptide, C34RE, were measured (Figure 3B,C). The spectrum of triN36e and C34RE mixture showed high molecular ellipticity as an absolute value comparable with that of triN36e alone. This supports the conclusion that C34RE interacts with tri36e and thereby induces a higher helical form as shown previously (*29*).

Mice were immunized with these synthetic gp41 mimetics and antibody production was successfully induced (the detailed titer increase in 5 weeks' immunization is given in the Supporting Information). Two out of three mice showed induction of antibodies against either antigen (N36RE or triN36e). Antibody titers and selectivity of antisera isolated from mice immunized with N36RE or triN36e were evaluated by serum titer ELISA against coated synthetic antigens. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Figure 4). The N36RE-induced antibody showed approximately 5 times higher affinity for N36RE than for triN36e, as 50% bound serum dilutions are 3.88×10^{-4} and 2.14×10^{-3} to N36RE and triN36e, respectively. It is noteworthy that the triN36e-induced antibody showed approximately 30 times higher preference in binding affinity for triN36e antigen than for N36RE (serum dilutions at 50% bound are 3.83×10^{-3} to N36RE and 1.33×10^{-4} to triN36e). Although this evaluation was not determined with purified mAbs, it is clear that the antibodies produced exploit a structural preference for antigens. The mechanism of induction

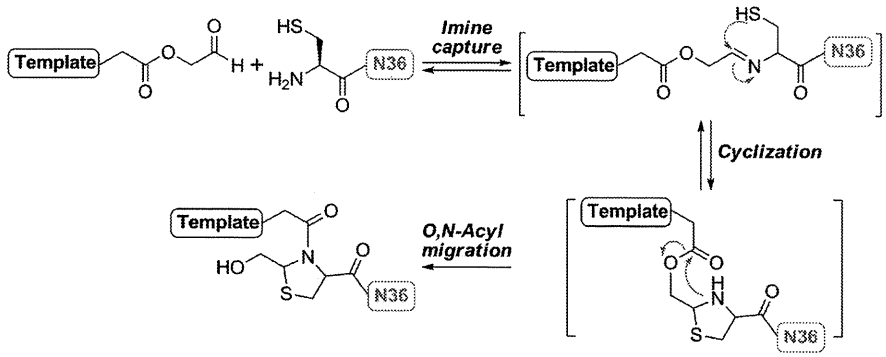


Figure 2. Reaction mechanisms of thiazolidine ligation utilized for assembly of N36RE helices on the template.

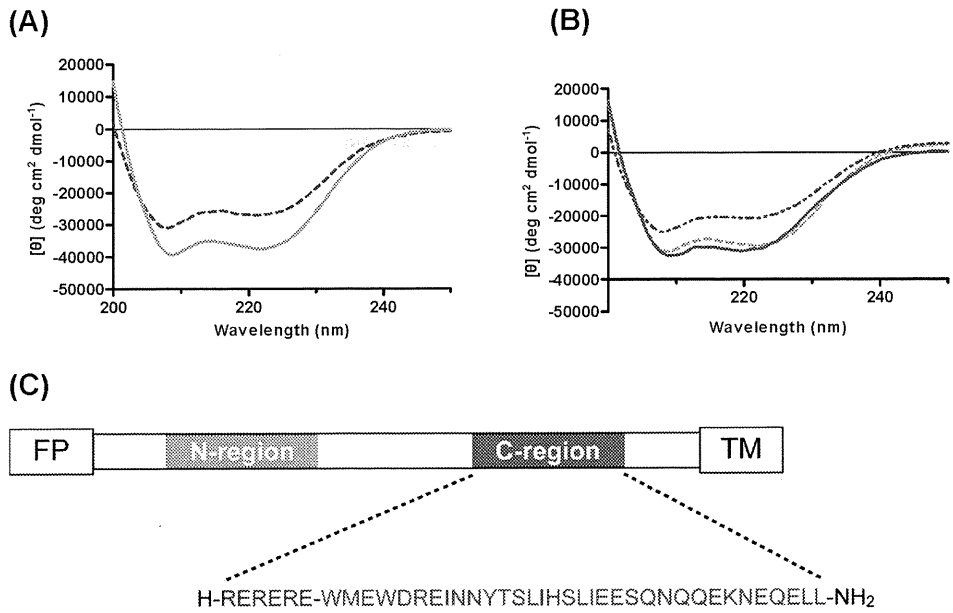


Figure 3. (A) Circular dichroism (CD) spectra of N36RE and triN36e. In the spectra, a blue dashed line and a green line show N36RE (monomer) and triN36e (trimer), respectively. Concentrations of the peptides are 10 and 3.3 μM for N36RE and triN36e, respectively. (B) CD spectra in the presence or absence of C34RE peptide. The spectra show the following: a dashed green line, triN36e; a dashed blue line, C34RE; a red line, triN36e+C34RE, respectively. The concentrations of peptides were as follows: triN36e (2.3 μM), C34-derived peptide C34RE (7 μM), and mixture of both peptides (3.5 μM each). (C) The amino acid sequence of C34RE described in single letters. FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively.

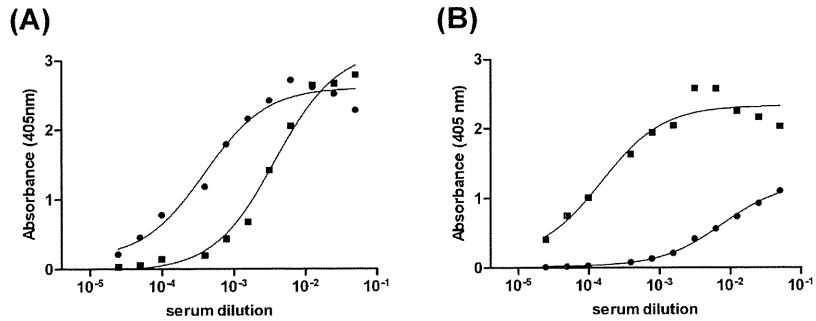


Figure 4. Serum titers of antibodies produced by N36 monomer and conformationally constrained N36 trimeric antigen. The titers were evaluated against N36RE (monomer) (A) and triN36e (trimer) (B). The plots indicate the results of sera obtained from N36RE-immunized mouse (•) and triN36e-immunized mouse (■).

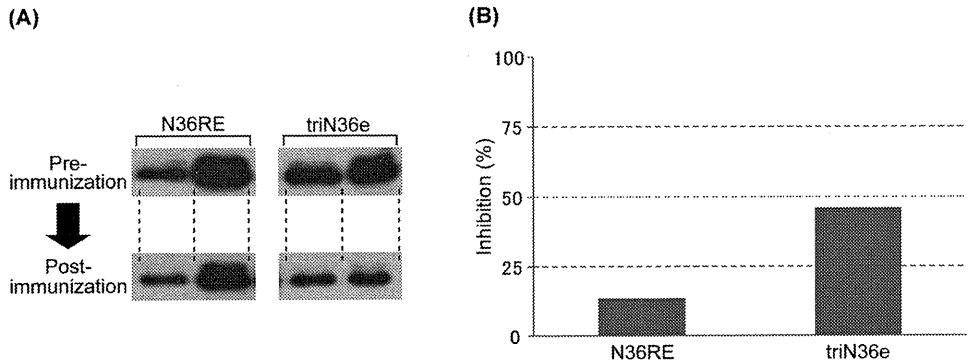


Figure 5. Determination of neutralization activity of the antibodies produced by immunization of peptidomimetic antigens. (A) Results of p24 assay to evaluate inhibition for HIV-1 infection by produced antibodies. Preimmunization sera were used as control. Experiments were duplicated. (B) Average % inhibition of p24 production calculated from the band intensities in panel (A).

of structure-specific antibody is still not clear, but the results could suggest the efficacy of producing antibodies with structural specificity and that the synthesis of structure-involving antigens is an effective strategy when higher specificity is required.

Neutralizing activity of sera against HIV-1 infection was assessed by p24 assays utilizing antisera from two mice that showed antibody production for each antigen (Figure 5). Sera

Table 1. Differences of α-Helicities between N36RE and triN36e Calculated from CD Spectra in Figure [3]

	$[\theta]_{222}$	$[\theta]_{222}/[\theta]_{208}$	α-helicity
N36RE	−30 957	0.87	73%
triN36e	−38 998	0.96	95%

Table 2. EC_{50} and CC_{50} Values Calculated from Inhibition Assays of Peptidomimetics

	AZT	triN36e	N36RE
EC_{50} (μM)[a]	0.047	0.49	1.4
CC_{50} (μM)[b]	>50	>1	>10

[a] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are based on the reduction of the viability of MT-4 cells. All data are the mean values for at least three experiments.

from mice immunized with the same antigen showed similar inhibitory activity against viral infection (12.5% and 14.8% for N36RE, 40.3% and 52.1% for triN36e). A trend was observed that the sera from triN36e immunization shows higher inhibition than those from N36RE immunization. This suggests that the synthetic antigen corresponding to the N36 trimeric form induces antibody with neutralization activity superior to that of the monomer peptide antigen and implies a restricted response of B-cells upon immunization to the trimeric form of N36RE. In order to assess the compatibility of induced antibodies in HIV-1 entry inhibition, the HIV-1 inhibitory activities of peptidomimetics (N36RE and triN36e) have been evaluated by viral infection and cytotoxicity assays. A C-terminal region peptide known as Enfuvirtide (T20, Roche/Trimeris) has been used clinically as a fusion inhibitor, and its success indicates that gp41-derived peptides might be potent inhibitors, useful against HIV-1 infection (*30*). In the development of anti-HIV peptides, several mimetics such as Enfuvirtide, CD4 binding site of gp120 (*31*), and protein-nucleic acid interactions (*32*), which disrupt protein−protein interactions, have been produced. As indicated in Table 2, N36 and triN36e showed modest inhibitory activity as reported in previous studies (*33*−*35*). The potency of triN36e was three times higher than that of N36RE indicating that the active structure of monomer N36RE is a trimeric form. Cytotoxicity of the antigens was not observed at concentrations of 1 μM of triN36e and 10 μM of N36RE.

CONCLUSIONS

In summary, a mimic of HIV-1 gp41-N36 designed as a new vaccine has been synthesized utilizing a novel template with three branched linkers of equal lengths. Thiazolidine-forming ligation attached the esteraldehyde of three-branched template with N-terminal cysteine of peptides in an aqueous medium. The resulting peptide antigen successfully induces antibodies with neutralization activity against HIV-1 infection. It is of special interest that the antibody produced acquires structural preference to antigen, which showed 30 times higher binding affinity for trimer than for monomer. This indicates the effectiveness of the design based on the structural dynamics of HIV-1 fusion mechanism of an antigen which could elicit neutralizing antibodies. In a design based on the N36 region of gp41, the exposed timing of epitopes is limited during HIV-1 entry (*36*), and carbohydrates, which could make accession of antibodies to epitopes difficult, are not associated with the amino acid residues of the native protein. These two advantages could further enhance the potential of a vaccine design based on the N36 region. During preparation of the manuscript, a new HIV vaccine strategy was reported by Burton's group (*37*). The report describes the importance of antibody recognition for the trimer form of surface protein. The trimer-specific antibodies indicate broad and potent neutralization. The gp41 trimer-form specific antibody produced in this study could also obtain the corresponding properties. The elucidation of antibody-producing mechanisms and epitope recognition mode of antibodies in antiserum during HIV-1 entry will be addressed in future studies.

ACKNOWLEDGMENT

The authors deeply thank Prof. K. Akiyoshi (Tokyo Medical and Dental Univ.) for allowing access to CD spectroporalimeter.

Supporting Information Available: HPLC chromatograms and NMR charts of compounds **3**, **5**, and **6**. Results of ESI-TOF-MS, and HPLC chromatograms of peptides N36RE, N36REGC, and triN36e. Results of serum titer ELISA of antisera collected during immunization. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Cabezas, E., Wang, M., Parren, P. W. H. I., Stanfield, R. L., and Satterthwait, A. C. (2000) A structure-based approach to a synthetic vaccine for HIV-1. *Biochemistry 39*, 14377–14391.

(2) Burton, D. R., Barbas, C. F., III, Persson, M. A. A., Koenig, S., Chanock, R. M., and Lerner, R. A. (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc. Natl. Acad. Sci. U.S.A. 88*, 10134–10137.

(3) Conley, A. J., Kessler, J. A. II, Boots, L. J., Tung, J. S., Arnold, B. A., Keller, P. M., Shaw, A. R., and Emini, R. A. (1994) Neutralization of divergent human immunodeficiency virus type 1 variants and primary isolates by IAM-41−2F5, an anti-gp41 human monoclonal antibody. *Proc. Natl. Acad. Sci. U.S.A. 91*, 3348–3352.

(4) Ofek, G., Tang, M., Sambor, A., Katinger, H., Mascola, J. R., Wyatt, R., and Kwong, P. D. (2004) Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. *J. Virol. 78*, 10724–10737.

(5) Alam, S. M., McAdams, M., Boren, D., Rak, M., Scearce, R. M., Gao, F., Camacho, Z. T., Gewirth, D., Kelsoe, G., Chen, P., and Haynes, B. F. (2007) The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. *J. Immunol. 178*, 4424–4435.

(6) Nelson, J. D., Brunel, F. M., Jensen, R., Crooks, E. T., Cardoso, R. M. F., Wang, M., Hessell, A., Wilson, I. A., Binley, J. M., Dawson, P. E., Burton, D. R., and Zwick, M. B. (2007) An affinity-enhanced neutralizing antibody against the membrane-proximal external region of human immunodeficiency virus type 1 gp41 recognizes an epitope between those of 2F5 and 4E10. *J. Virol. 81*, 4033–4043.

(7) Cardoso, R. M. F., Zwick, M. B., Stanfield, R. L., Kunert, R., Binley, J. M., Katinger, H., Burton, D. R., and Wilson, I. A. (2005) Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity 22*, 163–173.

(8) Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J. P., and Katinger, H. (1996) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J. Virol. 70*, 1100–1108.

(9) Pantophlet, R., Saphire, E. O., Poignard, P., Parren, P. W. H. I., Wilson, I. A., and Burton, D. R. (2003) Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. *J. Virol. 77*, 642–658.

(10) Sanders, R. W., Vesanen, M., Schuelke, N., Master, A., Schiffner, L., Kalyanaraman, R., Paluch, M., Berkhout, B., Maddon, P. J., Olson, W. C., Lu, M., and Moore, J. P. (2002) Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J. Virol. 76*, 8875–8889.

(11) Yang, X., Wyatt, R., and Sodroski, J. (2001) Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. *J. Virol. 75*, 1165–1171.
(12) Grundner, C., Mirzabekov, T., Sodroski, J., and Wyatt, R. (2002) Solid-phase proteoliposomes containing human immunodeficiency virus envelope glycoproteins. *J. Virol. 76*, 3511–3521.
(13) De Rosny, E., Vassell, R., Wingfield, R. T., Wild, C. T., and Weiss, C. D. (2001) Peptides corresponding to the heptad repeat motifs in the transmembrane protein (gp41) of human immunodeficiency virus type 1 elicit antibodies to receptor-activated conformations of the envelope glycoprotein. *J. Virol. 75*, 8859–8863.
(14) Tam, J. P., and Yu, Q. (2002) A facile ligation approach to prepare three-helix bundles of HIV fusion-state protein mimetics. *Org. Lett. 4*, 4167–4170.
(15) Xu, W., and Taylor, J. W. (2007) A template-assembled model of the N-peptide helix bundle from HIV-1 gp-41 with high affinity for C-peptide. *Chem. Biol. Drug Des. 70*, 319–328.
(16) Louis, J. M., Nesheiwat, I., Chang, L., Clore, G. M., and Bewlet, C. A. (2003) Covalent trimers of the internal N-terminal trimeric coiled-coil of gp41 and antibodies directed against them are potent inhibitors of HIV envelope-mediated cell fusion. *J. Biol. Chem. 278*, 20278–20285.
(17) Chen, Y.-H., Yang, J. T., and Chau, K. H. (1974) Determination of the helix and β form of proteins in aqueous solution by circular dichroism. *Biochemistry 13*, 3350–3359.
(18) Gans, P. J., Lyu, P. C., Manning, M. C., Woody, R. W., and Kallenbach, N. R. (1991) The helix-coil transition in heterogeneous peptides with specific side-chain interactions: theory and comparison with CD spectral data. *Biopolymers 13*, 1605–1614.
(19) Jackson, D. Y., King, D. S., Chmielewski, J., Singh, S., and Schultz, P. G. (1991) A general approach to the synthesis of short alpha-helical peptides. *J. Am. Chem. Soc. 113*, 9391–9392.
(20) Ohba, K., Ryo, A., Dewan, M. Z., Nishi, M., Naito, T., Qi, X., Inagaki, Y., Nagashima, Y., Tanaka, Y., Okamoto, T., Terashima, K., and Yamamoto, N. (2009) Follicular dendritic cells activate HIV-1 replication in monocytes/macrophages through a juxtacrine mechanism mediated by P-selectin glycoprotein ligand 1. *J. Immunol. 183*, 524–532.
(21) Liu, J., Shu, W., Fagan, M. B., Nunberg, J. H., and Lu, H. (2001) Structural and functional analysis of the HIV gp41 core containing an Ile573 to Thr substitution: implications for membrane fusion. *Biochemistry 40*, 2797–2807.
(22) Liu, C. F., and Tam, J. P. (1994) Peptide segment ligation strategy without use of protecting groups. *Proc. Natl. Acad. Sci. U.S.A. 91*, 6584–6588.
(23) Tam, J. P., and Miao, Z. (1999) Stereospecific pseudoproline ligation of N-terminal serine, threonine, or cysteine-containing unprotected peptides. *J. Am. Chem. Soc. 121*, 9013–9022.
(24) Tam, J. P., Yu, Q., and Yang, J.-L. (2001) Tandem ligation of unprotected peptides through thiaprolyl and cysteinyl bonds in water. *J. Am. Chem. Soc. 123*, 2487–94.
(25) Eom, K. D., Miao, Z., Yang, J.-L., and Tam, J. P. (2003) Tandem ligation of multipartite peptides with cell-permeable activity. *J. Am. Chem. Soc. 125*, 73–82.
(26) Sadler, K., Zhang, Y., Xu, J., Yu, Q., and Tam, J. P. (2008) Quaternary protein mimetics of gp41 elicit neutralizing antibodies against HIV fusion-active intermediate state. *Biopolym. (Pept. Sci.) 90*, 320–329.
(27) Bychkova, V. E., Dujsekina, A. E., Klenin, S. I., Tiktopulo, E. I., Uversky, V. N., and Ptitsyn, O. B. (1996) Molten globule-like state of cytochrome *c* under conditions simulating those near the membrane surface. *Biochemistry 35*, 6058–6063.
(28) Nishi, K., Komine, Y., Sakai, N., Maruyama, T., and Otagiri, M. (2005) Cooperative effect of hydrophobic and electrostatic forces on alcohol-induced α-helix formation of α_1-acid glycoprotein. *FEBS Lett. 579*, 3596–3600.
(29) Chan, D. C., Chutkowski, C. T., and Kim, P. S. (1998) Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proc. Natl. Acad. Sci. U.S.A. 95*, 15613–15617.
(30) Liu, S., Jing, W., Cheng, B., Lu, H., Sun, J., Yan, X., Niu, J., Farmar, J., Wu, S., and Jiang, S. (2007) HIV gp41 C-terminal heptad repeat contains multifunctional domains: relation to mechanism of action of anti-HIV peptides. *J. Biol. Chem. 282*, 9612–9620.
(31) Franke, R., Hirsch, T., Overwin, H., and Eichler, J. (2007) Synthetic mimetics of the CD4 binding site of HIV-1 gp120 for the design of immunogens. *Angew. Chem., Int. Ed. 46*, 1253–1255.
(32) Robinson, J. A. (2008) β-hairpin peptidomimetics: design, structures and biological activities. *Acc. Chem. Res. 41*, 1278–1288.
(33) Lu, M., Ji, H., and Shen, S. (1999) Subdomain folding and biological activity of the core structure from human immunodeficiency virus type 1 gp41: implications for viral membrane fusion. *J. Virol. 73*, 4433–4438.
(34) Eckert, D. M., and Kim, P. S. (2001) Design of potent inhibitors of HIV-1 entry from the gp41 N-peptide region. *Proc. Natl. Acad. Sci. U.S.A. 98*, 11187–11192.
(35) Bianchi, E., Finotto, M., Ingallinella, P., Hrin, R., Carella, A. V., Hous, X. S., Schleif, W. A., and Miller, M. D. (2005) Covalent stabilization of coiled coils of the HIV gp41 N region yields extreamely potent and broad inhibitors of viral infection. *Proc. Natl. Acad. Sci. U.S.A. 102*, 12903–12908.
(36) Zwick, M. B., Saphire, E. O., and Burton, D. R. (2004) gp41: HIV's shy protein. *Nat. Med. 10*, 133–134.
(37) Walker, L. M., Phogat, S. K., Chan-Hui, P.-Y., Wagner, D., Phung, P., Goss, J. L., Wrin, T., Simek, M. D., Fling, S., Mitcham, J. L., Lehrman, J. K., Priddy, F. H., Olsen, O. A., Frey, S. M., Hammond, P. W., Kaminsky, S., Zamb, T., Moyle, M., Koff, W. C., Poignard, P., and Burton, D. R. (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science 326*, 285–289.

BC900502Z

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

Takeo Kuwata,[1] Yuko Katsumata,[1] Kaori Takaki,[1] Tomoyuki Miura,[2] and Tatsuhiko Igarashi[2]

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

[1]Priority Organization for Innovation and Excellence, Kumamoto University, Honjyo, Kumamoto, Japan.
[2]Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto, Japan.

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′)

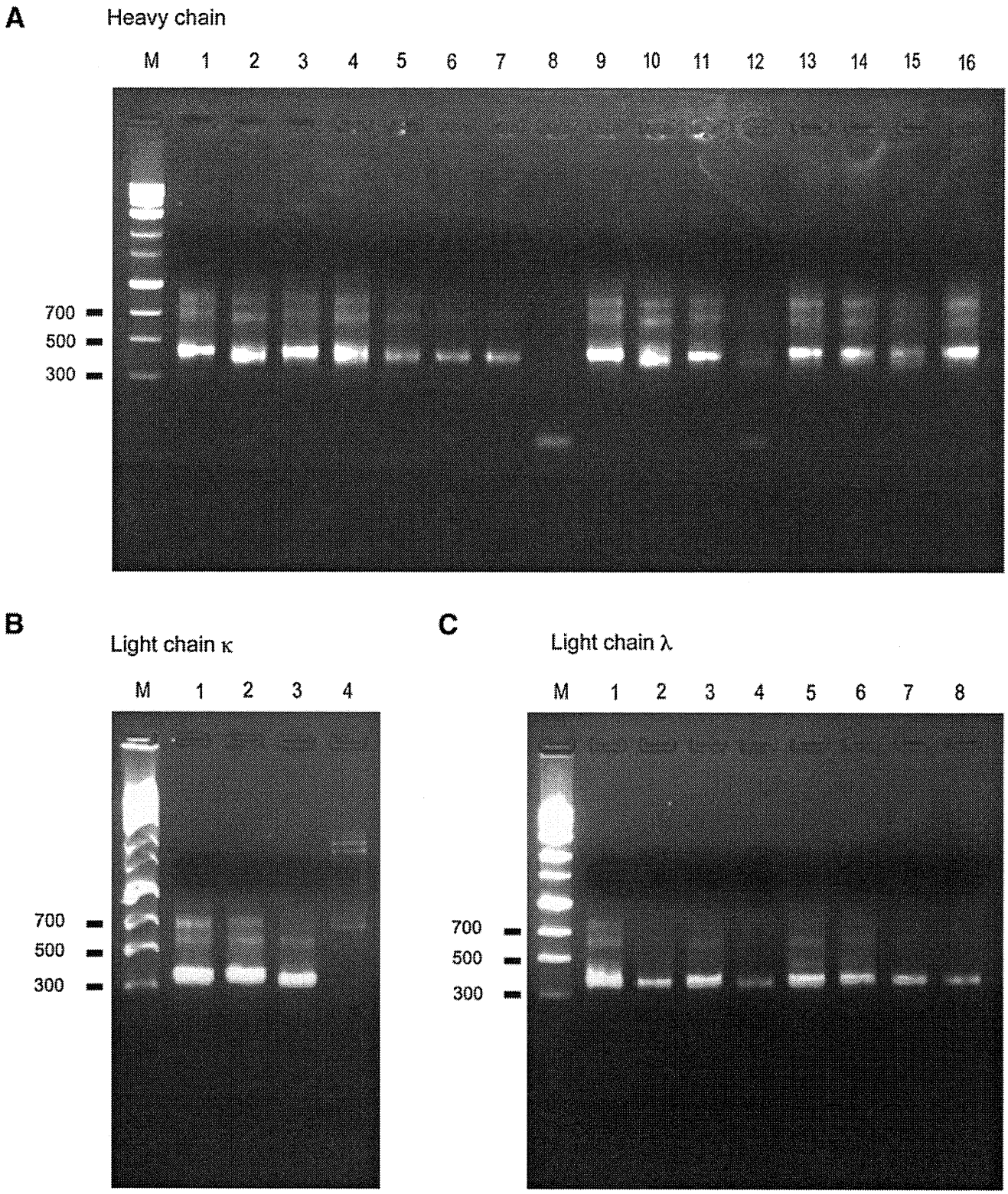


FIG. 1. Ig heavy chain and light chain κ and λ variable regions were successfully amplified by PCR using primers shown in Table 1. **(A)** The Ig heavy chain variable region was amplified using the following primer pairs: lane 1, RhFabVH17-F and RhFabVHJ1-B; 2, RhFabVH17-F and RhFabVHJ2-B; 3, RhFabVH17-F 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

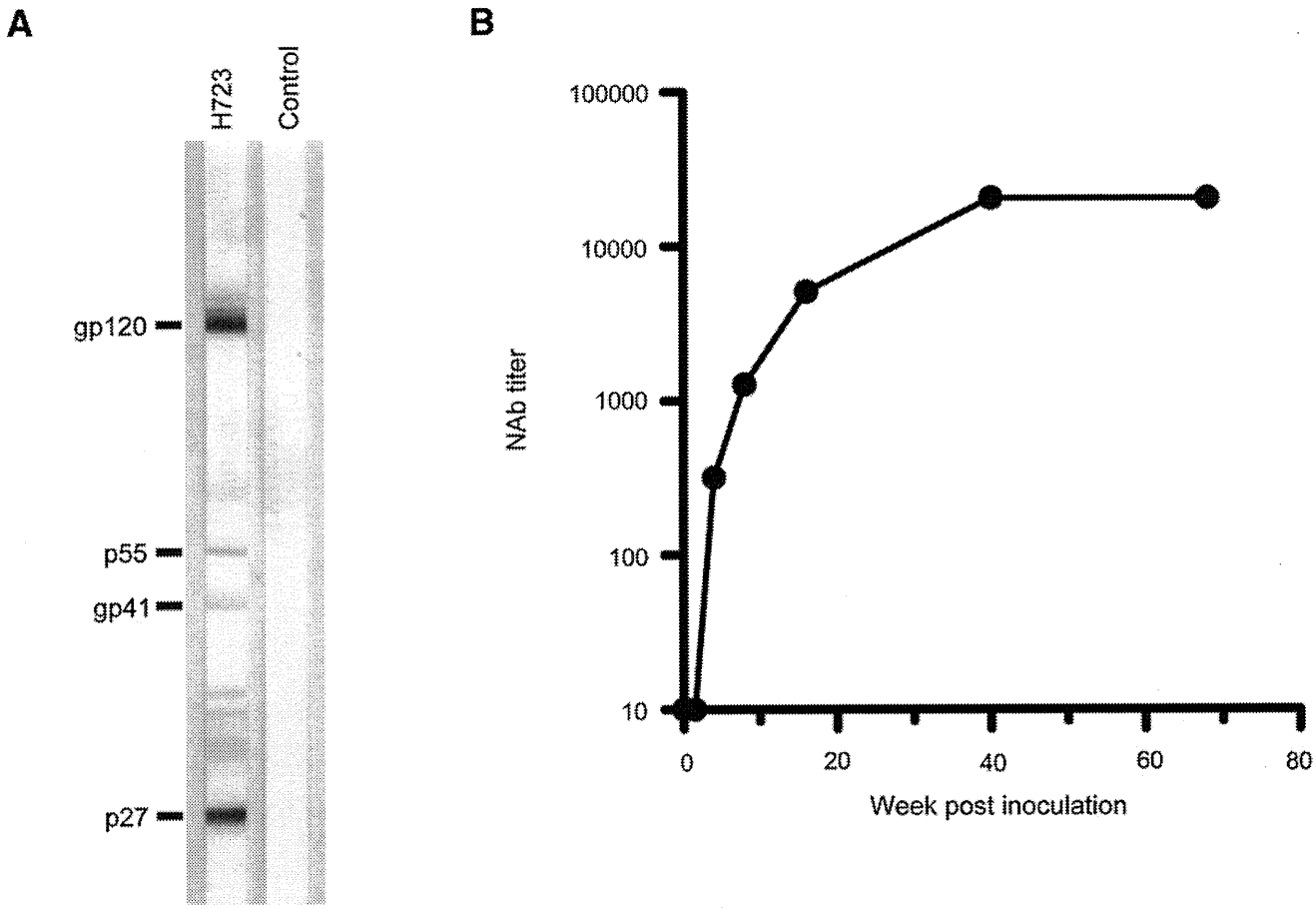


FIG. 2. Antibody response in SIVsmH635FC-infected macaque H723. **(A)** Western blot analysis showed that antibodies in plasma from H723 at death recognized Env gp120 and gp41 and Gag p55 and p27. **(B)** H723 maintained a high NAb titer during the course of infection. Neutralizing titer was determined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

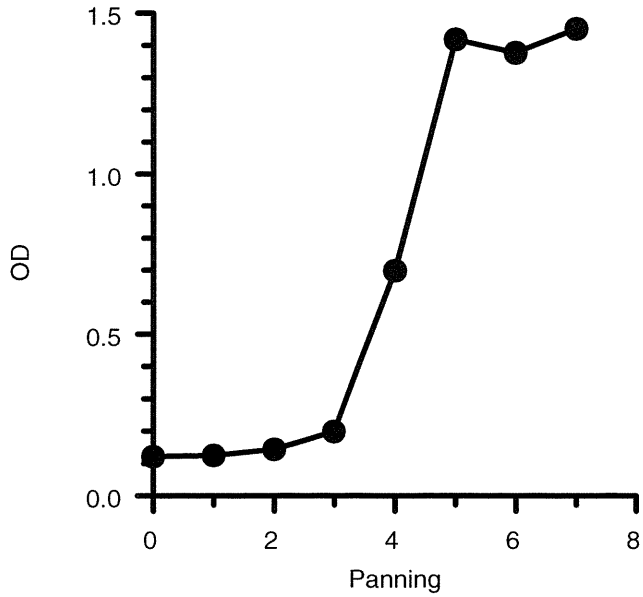


FIG. 3. Reactivity of phage to SIV Ag increased from the fourth round of panning. Phage samples that were eluted after panning were examined by ELISA for their ability to bind SIV proteins.

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.

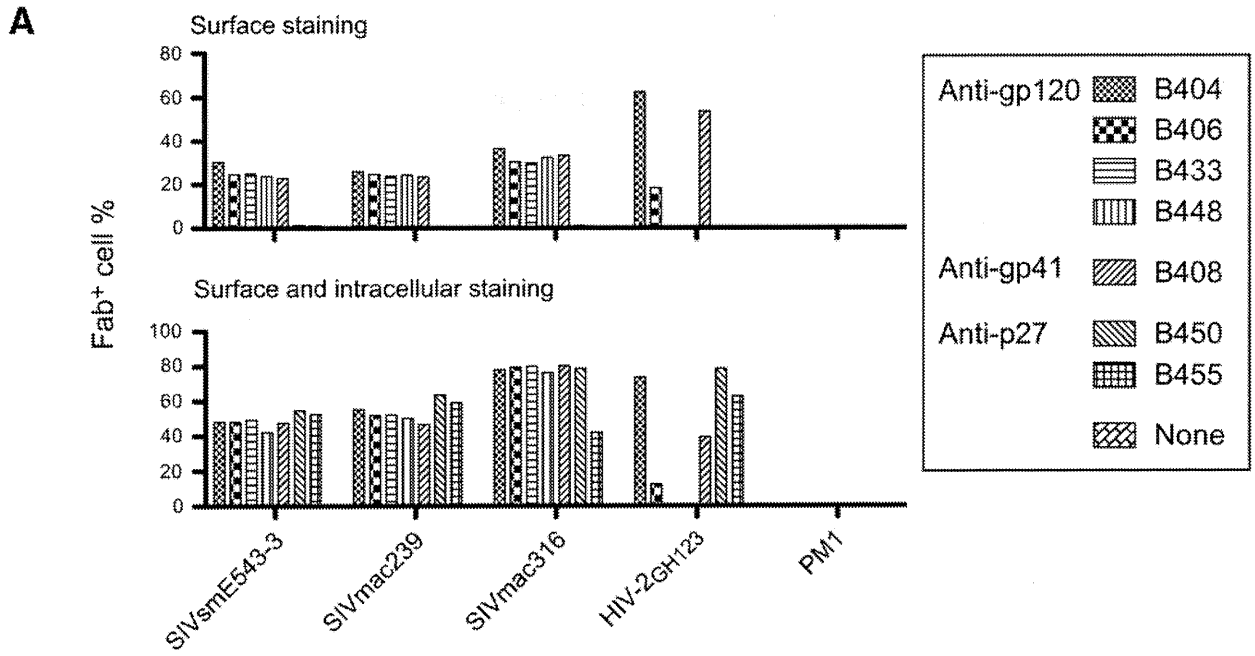
Selection of SIV-specific Fab clones by panning

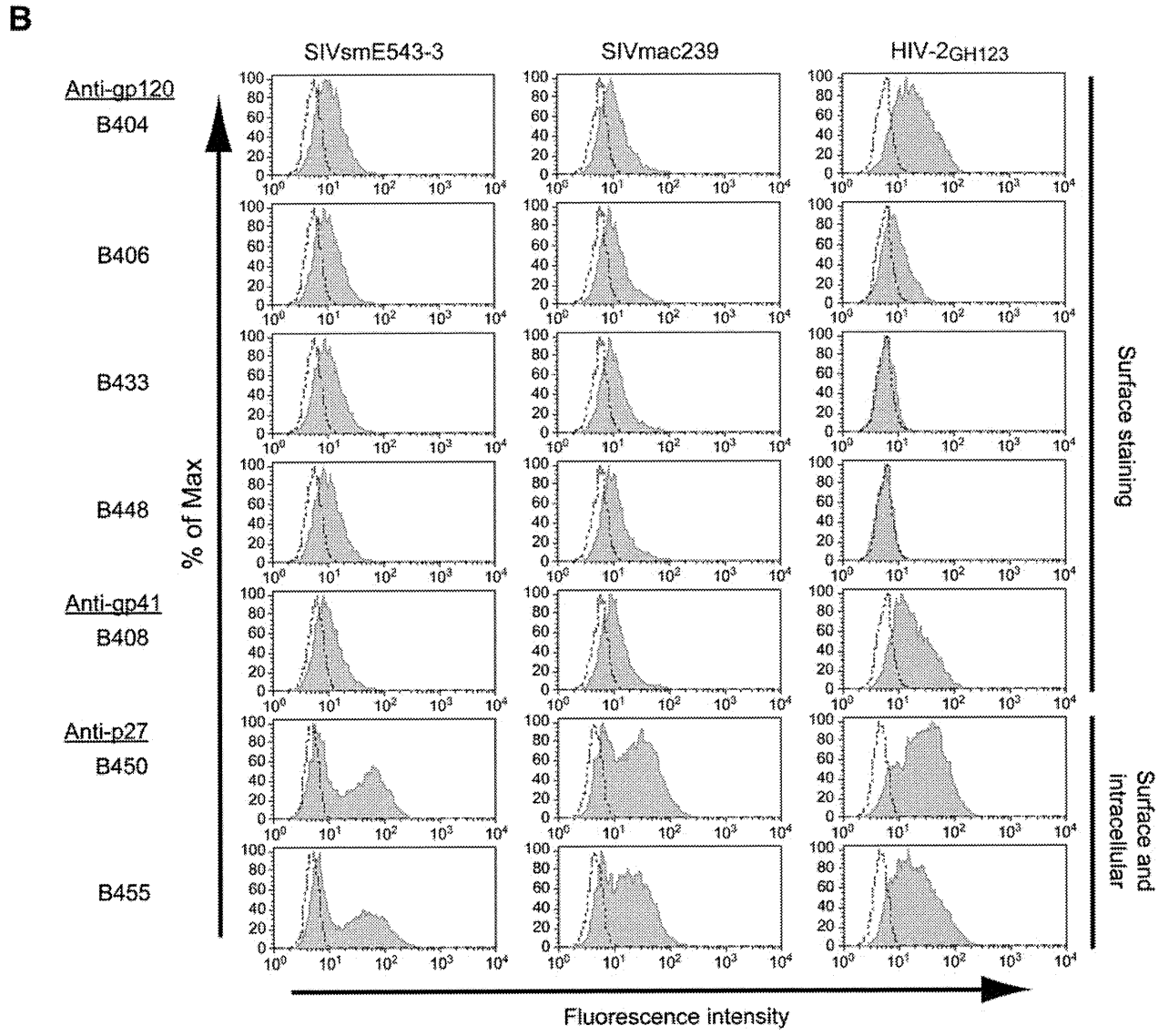
Biopanning was performed on a whole, Triton X-100-disrupted SIVsmE543-3 Ag-coated 96-well plate. The ability of phage to bind SIV Ag increased from the fourth round of panning, indicating successful selection of SIV-specific Fab (Fig. 3). Phagemid DNA was prepared from the fourth and fifth round of panning, 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
Surface staining
Surface and intracellular
Fluorescence intensity

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]

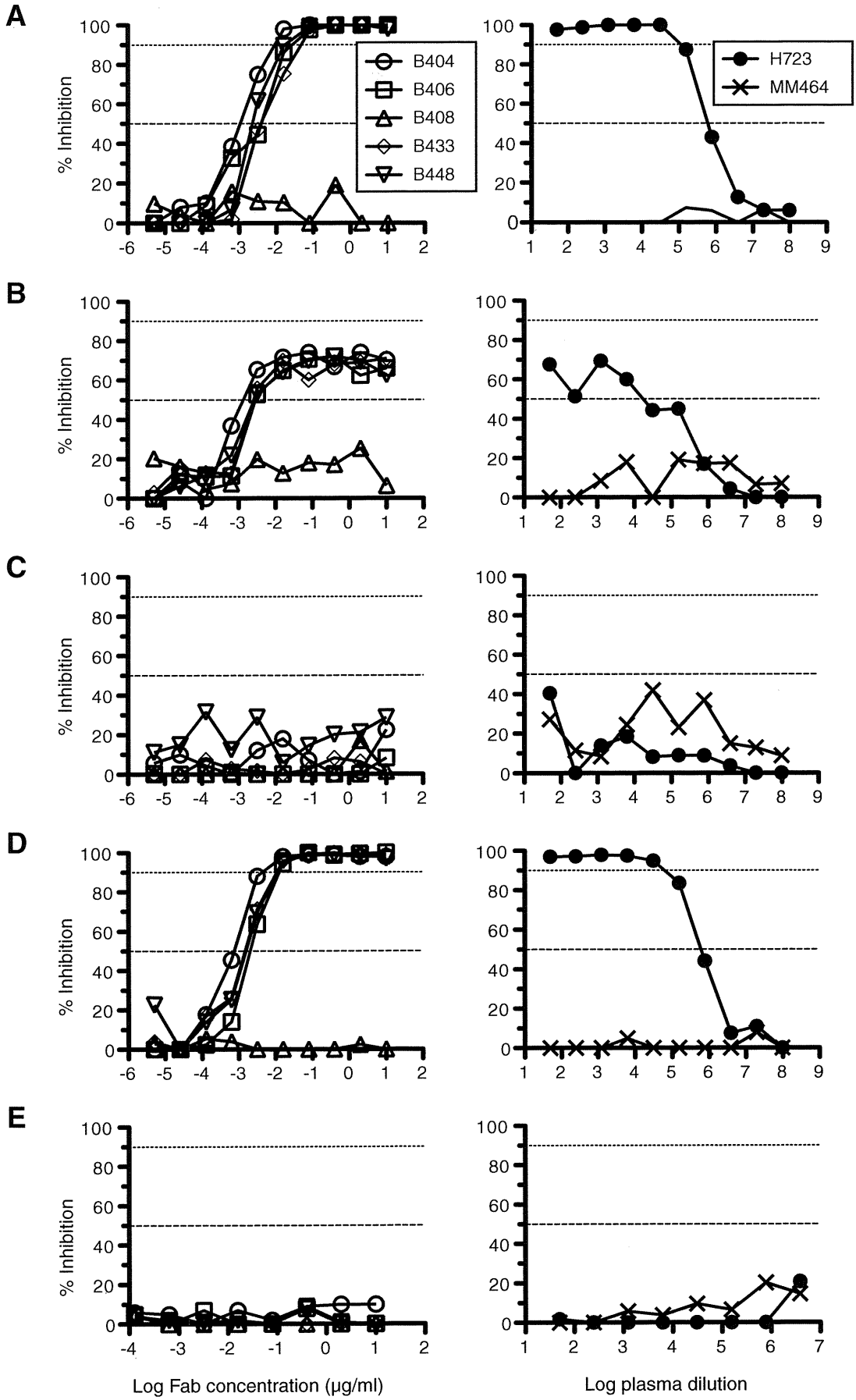
SIVmac239, another heterologous strain, and HIV-2_{GH123} were not neutralized by any Fab (Fig. 5C and E). HIV-2_{GH123} was not neutralized, perhaps because of its low cross-reactivity, though B404 bound HIV-2_{GH123} (Fig. 4). Unsuccessful neutralization of SIVmac239 may be related to the Env structure, 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

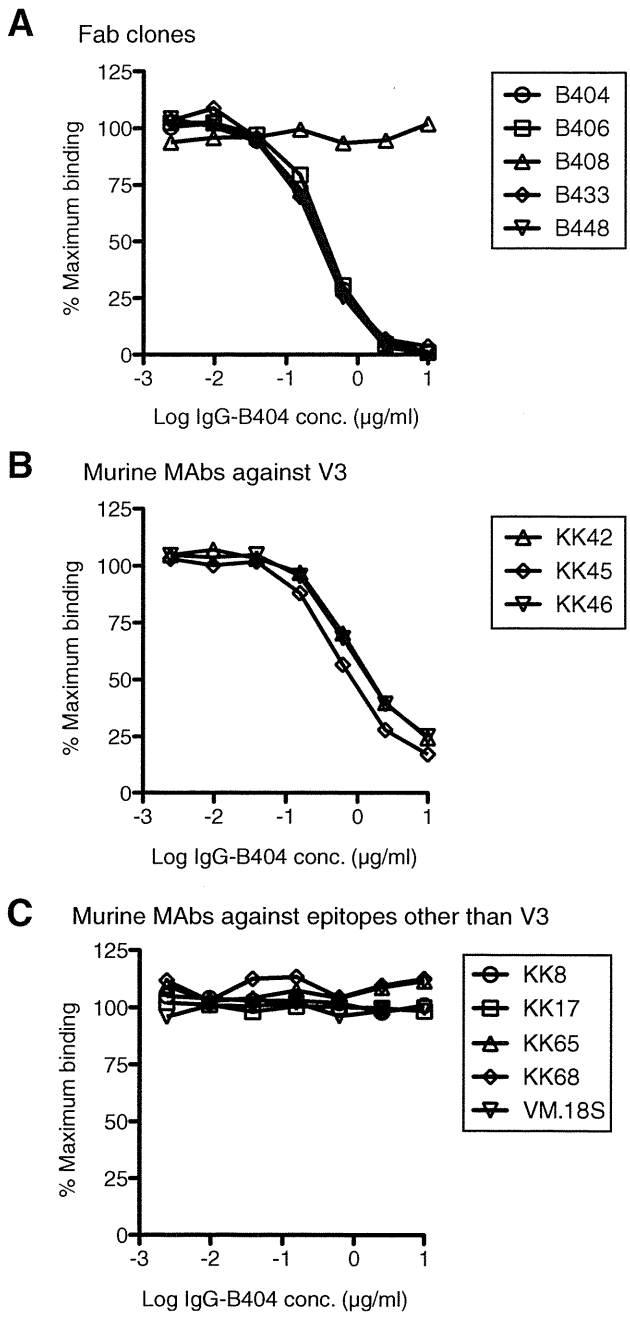


FIG. 6. Identification of epitope specificity of anti-gp120 Fab clones by competition ELISA. **(A)** IgG-B404 inhibited the binding of anti-gp120 Fabs, B404, B406, B433, and B448, but did not inhibit anti-gp41 Fab B406. **(B)** IgG-B404 competed with the binding of murine MAbs, KK42, KK45, and KK46, 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.

References

1. Igarashi T, Brown C, Azadegan A, *et al.*: Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. Nat Med 1999;5(2):211–216.
2. Shibata R, Igarashi T, Haigwood N, *et al.*: Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med 1999;5(2):204–210.
3. Veazey RS, Shattock RJ, Pope M, *et al.*: Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 2003;9(3): 343–346.
4. Hessell AJ, Rakasz EG, Poignard P, *et al.*: Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathog 2009;5(5):e1000433.
5. Mascola JR, Stiegler G, VanCott TC, *et al.*: Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 2000;6(2):207–210.
6. D'Souza MP, Livnat D, Bradac JA, and Bridges SH: Evaluation of monoclonal antibodies to human immunodeficiency virus type 1 primary isolates by neutralization assays: Performance criteria for selecting candidate antibodies for clinical trials. AIDS Clinical Trials Group Antibody Selection Working Group. J Infect Dis 1997;175(5):1056–1062.
7. Walker LM, Phogat SK, Chan-Hui PY, *et al.*: Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 2009;326(5950):285–289.
8. Saphire EO, Parren PW, Pantophlet R, *et al.*: Crystal structure of a neutralizing human IGG against HIV-1: A template for vaccine design. Science 2001;293(5532):1155–1159.
9. Karlsson Hedestam GB, Fouchier RA, Phogat S, Burton DR, Sodroski J, and Wyatt RT: The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nat Rev Microbiol 2008;6(2):143–155.
10. Li Y, Migueles SA, Welcher B, *et al.*: Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat Med 2007;13(9):1032–1034.
11. Haynes BF, Fleming J, St Clair EW, *et al.*: Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science 2005;308(5730):1906–1908.
12. Ofek G, McKee K, Yang Y, *et al.*: Relationship between antibody 2F5 neutralization of HIV-1 and hydrophobicity of its heavy chain third complementarity-determining region. J Virol 2010;84(6):2955–2962.

13. Poignard P, Saphire EO, Parren PW, and Burton DR: gp120: Biologic aspects of structural features. Annu Rev Immunol 2001;19:253–274.
14. Hirsch VM and Lifson JD: Simian immunodeficiency virus infection of monkeys as a model system for the study of AIDS pathogenesis, treatment, and prevention. Adv Pharmacol 2000;49:437–477.
15. Hirsch V, Adger-Johnson D, Campbell B, *et al.*: A molecularly cloned, pathogenic, neutralization-resistant simian immunodeficiency virus, SIVsmE543-3. J Virol 1997;71(2):1608–1620.
16. Johnson WE, Sanford H, Schwall L, *et al.*: Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. J Virol 2003; 77(18):9993–10003.
17. Means RE, Matthews T, Hoxie JA, Malim MH, Kodama T, and Desrosiers RC: Ability of the V3 loop of simian immunodeficiency virus to serve as a target for antibody-mediated neutralization: Correlation of neutralization sensitivity, growth in macrophages, and decreased dependence on CD4. J Virol 2001;75(8):3903–3915.
18. Samuelsson A, Chiodi F, Ohman P, Putkonen P, Norrby E, and Persson MA: Chimeric macaque/human Fab molecules neutralize simian immunodeficiency virus. Virology 1995; 207(2):495–502.
19. Chassagne S, Laffly E, Drouet E, Herodin F, Lefranc MP, and Thullier P: A high-affinity macaque antibody Fab with human-like framework regions obtained from a small phage display immune library. Mol Immunol 2004;41(5):539–546.
20. Glamann J, Burton DR, Parren PW, *et al.*: Simian immunodeficiency virus (SIV) envelope-specific Fabs with high-level homologous neutralizing activity: Recovery from a long-term-nonprogressor SIV-infected macaque. J Virol 1998; 72(1):585–592.
21. Druar C, Saini SS, Cossitt MA, *et al.*: Analysis of the expressed heavy chain variable-region genes of Macaca fascicularis and isolation of monoclonal antibodies specific for the Ebola virus' soluble glycoprotein. Immunogenetics 2005;57(10):730–738.
22. Cole KS, Alvarez M, Elliott DH, *et al.*: Characterization of neutralization epitopes of simian immunodeficiency virus (SIV) recognized by rhesus monoclonal antibodies derived from monkeys infected with an attenuated SIV strain. Virology 10 2001;290(1):59–73.
23. Robinson JE, Cole KS, Elliott DH, *et al.*: Production and characterization of SIV envelope-specific rhesus monoclonal antibodies from a macaque asymptomatically infected with a live SIV vaccine. AIDS Res Hum Retroviruses 1998;14(14): 1253–1262.
24. Lusso P, Cocchi F, Balotta C, *et al.*: Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): Failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. J Virol 1995;69(6):3712–3720.
25. Takeuchi Y, McClure MO, and Pizzato M: Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research. J Virol 2008;82(24):12585–12588.
26. Wei X, Decker JM, Liu H, *et al.*: Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob Agents Chemother 2002;46(6):1896–1905.
27. Derdeyn CA, Decker JM, Sfakianos JN, *et al.*: Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J Virol 2000;74(18):8358–8367.
28. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, and Kabat D: Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J Virol 1998;72(4):2855–2864.
29. Morner A, Bjorndal A, Albert J, *et al.*: Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. J Virol 1999;73(3):2343–2349.
30. DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH, and Calos MP: Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol Cell Biol 1987;7(1): 379–387.
31. Kuwata T, Byrum R, Whitted S, *et al.*: A rapid progressor-specific variant clone of simian immunodeficiency virus replicates efficiently in vivo only in the absence of immune responses. J Virol 2007;81(17):8891–8904.
32. Kuwata T, Dehghani H, Brown CR, *et al.*: Infectious molecular clones from a simian immunodeficiency virus-infected rapid-progressor (RP) macaque: Evidence of differential selection of RP-specific envelope mutations in vitro and in vivo. J Virol 2006;80(3):1463–1475.
33. Kuwata T, Nishimura Y, Whitted S, *et al.*: Association of progressive CD4(+) T cell decline in SIV infection with the induction of autoreactive antibodies. PLoS Pathog 2009;5(4): e1000372.
34. Kestler H, Kodama T, Ringler D, *et al.*: Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science 1990;248(4959):1109–1112.
35. Mori K, Ringler DJ, Kodama T, and Desrosiers RC: Complex determinants of macrophage tropism in env of simian immunodeficiency virus. J Virol 1992;66(4):2067–2075.
36. Shibata R, Miura T, Hayami M, *et al.*: Mutational analysis of the human immunodeficiency virus type 2 (HIV-2) genome in relation to HIV-1 and simian immunodeficiency virus SIV (AGM). J Virol 1990;64(2):742–747.
37. Barbas CF, Scott JM, Silverman G, and Burton DR: *Phage Display: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
38. Robinson JE, Holton D, Liu J, McMurdo H, Murciano A, and Gohd R: A novel enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HIV-1 envelope glycoproteins based on immobilization of viral glycoproteins in microtiter wells coated with concanavalin A. J Immunol Methods 1990;132(1):63–71.
39. Kent KA, Gritz L, Stallard G, *et al.*: Production and of monoclonal antibodies to simian immunodeficiency virus envelope glycoproteins. AIDS 1991;5(7):829–836.
40. Kent KA, Rud E, Corcoran T, *et al.*: Identification of two neutralizing and 8 non-neutralizing epitopes on simian immunodeficiency virus envelope using monoclonal antibodies. AIDS Res Hum Retroviruses 1992;8(6):1147–1151.
41. Lefranc MP, Giudicelli V, Ginestoux C, *et al.*: IMGT, the international ImMunoGeneTics information system. Nucleic Acids Res 2009;37(Database issue):D1006–1012.
42. Ourmanov I, Kuwata T, Goeken R, *et al.*: Improved survival in rhesus macaques immunized with modified vaccinia virus Ankara recombinants expressing simian immunodeficiency virus envelope correlates with reduction in memory CD4+ T-cell loss and higher titers of neutralizing antibody. J Virol 2009;83(11):5388–5400.
43. Andris JS, Miller AB, Abraham SR, *et al.*: Variable region gene segment utilization in rhesus monkey hybridomas

producing human red blood cell-specific antibodies: Predominance of the VH4 family but not VH4-21 (V4-34). Mol Immunol 1997;34(3):237–253.
44. Bible JM, Howard W, Robbins H, and Dunn-Walters DK: IGHV1, IGHV5 and IGHV7 subgroup genes in the rhesus macaque. Immunogenetics 2003;54(12):867–873.
45. Ermert K, Mitlohner H, Schempp W, and Zachau HG: The immunoglobulin kappa locus of primates. Genomics 1995; 25(3):623–629.
46. Helmuth EF, Letvin NL, and Margolin DH: Germline repertoire of the immunoglobulin V(H)3 family in rhesus monkeys. Immunogenetics 2000;51(7):519–527.
47. Howard WA, Bible JM, Finlay-Dijsselbloem E, Openshaw S, and Dunn-Walters DK: Immunoglobulin light-chain genes in the rhesus macaque I: Kappa light-chain germline sequences for subgroups IGKV1, IGKV and IGKV3. Immunogenetics 2005;57(3–4):210–218.
48. Howard WA, Bible JM, Finlay-Dijsselbloem E, Openshaw S, and Dunn-Walters DK: Immunoglobulin light-chain genes in the rhesus macaque II: Lambda light-chain germline sequences for subgroups IGLV1, IGLV2, IGLV3, IGLV4 and IGLV5. Immunogenetics 2005;57(9): 655–664.
49. Link JM, Hellinger MA, and Schroeder HW Jr: The rhesus monkey immunoglobulin IGHD and IGHJ germline repertoire. Immunogenetics 2002;54(4):240–250.
50. Link JM, Larson JE, and Schroeder HW: Despite extensive similarity in germline DH and JH sequence, the adult rhesus macaque CDR-H3 repertoire differs from human. Mol Immunol 2005;42(8):943–955.
51. Scinicariello F, Engleman CN, Jayashankar L, McClure HM, and Attanasio R: Rhesus macaque antibody molecules: Sequences and heterogeneity of alpha and gamma constant regions. Immunology 2004;111(1):66–74.
52. Gibbs RA, Rogers J, Katze MG, *et al.*: Evolutionary and biomedical insights from the rhesus macaque genome. Science 2007;316(5822):222–234.

Address correspondence to:
Takeo Kuwata
Priority Organization for Innovation and Excellence
Kumamoto University
Honjyo-Kyoyo-to Rm208
2-2-1 Honjyo
Kumamoto 860-0811
Japan
E-mail: tkuwata@kumamoto-u.ac.jp

Virology 399 (2010) 134–143

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

In vivo analysis of a new R5 tropic SHIV generated from the highly pathogenic SHIV-KS661, a derivative of SHIV-89.6

Kenta Matsuda, Katsuhisa Inaba, Yoshinori Fukazawa, Megumi Matsuyama, Kentaro Ibuki, Mariko Horiike, Naoki Saito, Masanori Hayami, Tatsuhiko Igarashi, Tomoyuki Miura *

Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoinkawaramachi, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 9 November 2009
Returned to author for revision
14 December 2009
Accepted 5 January 2010
Available online 27 January 2010

Keywords:
AIDS
SHIV
CCR5 tropic
Mutagenesis
V3 region

ABSTRACT

Although X4 tropic SHIVs have been studied extensively, they show distinct infection phenotypes from those of R5 tropic viruses, which play an important role in HIV-1 transmission and pathogenesis. To augment the variety of R5 tropic SHIVs, we generated a new R5 tropic SHIV from the highly pathogenic X4 tropic SHIV-KS661, a derivative of SHIV-89.6. Based on consensus amino acid alignment analyses of subtype B R5 tropic HIV-1, five amino acid substitutions in the third variable region successfully changed the secondary receptor preference from X4 to R5. Improvements in viral replication were observed in infected rhesus macaques after two passages, and reisolated virus was designated SHIV-MK38. SHIV-MK38 maintained R5 tropism through *in vivo* passages and showed robust replication in infected monkeys. Our study clearly demonstrates that a minimal number of amino acid substitutions in the V3 region can alter secondary receptor preference and increase the variety of R5 tropic SHIVs.

Introduction

Simian immunodeficiency virus (SIV) macaque models for AIDS have been used extensively to elucidate the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection. Although SIV is an excellent model virus that has contributed to various virological discoveries, SIV has many limitations as an HIV-1 model. Because the antigenicity of SIV is different from that of HIV-1, it is difficult to evaluate HIV-1 vaccines in animal models by employing SIV as a challenge virus. This is especially true for evaluating the induction of neutralizing antibodies by HIV-1 vaccine candidates (Baba et al., 2000; Dey et al., 2009; Mascola et al., 2000). In addition to CCR5, SIV utilizes secondary receptors such as GPR1, GPR15 (Bob), and STRL-33 (Bonzo), which are scarcely used by HIV-1 (Clapham and McKnight, 2002). Although there have been no reports that have directly demonstrated the significance of these receptors for *in vivo* pathogenesis, possible influences of these minor receptors cannot be denied.

To supplement the limitations of the SIV model, a simian and human immunodeficiency virus (SHIV) macaque model has been generated. SHIVs were constructed by exchanging the envelope gene and other accessory genes of SIV with that of HIV-1 (Shibata et al., 1991). Therefore, SHIVs share the same envelope antigenicity and receptor usage with HIV-1. In early studies of HIV-1, isolated viruses were mostly X4 or dual tropic because they were isolated from AIDS patients using T-cell lines expressing CXCR4. Because envelope genes from X4 or dual tropic viruses were introduced to generate the chimeric virus, most SHIVs utilize CXCR4 as a secondary receptor. X4 tropic viruses infect distinct subsets of lymphocytes and the mode of viral replication during the acute phase of infection is different from that of R5 tropic viruses (Nishimura et al., 2004). During the acute phase of infection, X4 tropic SHIVs rapidly deplete circulating CD4 positive (+) T cells (Reimann et al., 1996; Sadjadpour et al., 2004). Most infected monkeys fail to seroconvert, because rapid depletion of helper T cells typically occurs within 4 weeks of infection. In contrast, R5 tropic viruses do not show such a catastrophic reduction in CD4+ T cells. The phenotypes observed during X4 SHIV infection are rare during actual HIV-1 infection, and it has been suggested that R5 tropic viruses are mainly involved in HIV-1 transmission and pathogenesis (Margolis and Shattock, 2006). Therefore, there is a demand for R5 tropic SHIVs in this field of research.

There are some R5 tropic SHIVs that have already been used in various experiments, including analyses on the efficacy of broadly neutralizing antibodies (Hessell et al., 2009). Due to the paucity of available R5 tropic SHIVs, however, it is difficult to conduct comparative analyses on the efficacy of neutralizing antibodies between different strains of SHIVs. *In vivo* analyses of neutralizing antibodies should be conducted with more than one or even a mixture of several strains of R5 tropic virus to reflect the wide variety of HIV-1 envelope genes that are found worldwide. Therefore, our primary aim

* Corresponding author. Mailing address: Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoinkawaramachi, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81 75 761 9335.
E-mail address: tmiura@virus.kyoto-u.ac.jp (T. Miura).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.01.008

was to generate a new R5 tropic SHIV, which carries a different *env* from that of other existing R5 SHIVs.

Currently available R5 SHIVs were constructed by introducing the envelope gene and other accessory genes from R5 tropic HIV-1 into the SIV backbone (Humbert et al., 2008; Luciw et al., 1995). There is one report that demonstrated the construction of an R5 tropic SHIV by exchanging the whole third variable region (V3) of an X4 tropic SHIV with that of an R5 SHIV (Ho et al., 2005). This study clearly indicated that the V3 region of the envelope gene determines the secondary receptor preference *in vivo*. Although other studies have indicated that there are specific amino acids within the V3 region that are responsible for receptor preference (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004), there have been no reports demonstrating the generation of R5 tropic SHIV by the introduction of specific amino acid substitutions to the V3 region. Therefore, our secondary aim in this study was to alter the receptor usage of a well-studied X4 tropic SHIV by introducing a minimal number of amino acid substitutions in the *env* V3 region. The consensus amino acid alignment of subtype B R5 tropic HIV-1, which is strongly correlated with secondary receptor usage (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004), was introduced to the V3 region of a highly pathogenic SHIV-KS661 that possesses the typical infection phenotype of X4 tropic SHIV (Fukazawa et al., 2008; Miyake et al., 2006). SHIV-KS661 is a molecular clone constructed from the consensus sequence of SHIV-C2/1 (Gen Bank accession number AF21718) (Shinohara et al., 1999), a derivative of the non-pathogenic SHIV-89.6

Results

Generation of R5 tropic SHIV-MK1 from the highly pathogenic X4 tropic SHIV-KS661

The X4 tropic virus SHIV-KS661, a derivative of SHIV-89.6, depletes CD4+ T lymphocytes in systemic tissues within weeks of infection and causes AIDS-like symptoms in macaque monkeys (Fukazawa et al., 2008; Miyake et al., 2006). To convert the virus into an R5 tropic virus, we introduced five amino acid substitutions in the V3 region of SHIV-KS661 by site-directed mutagenesis. The positions of the substitutions were selected using information from alignment of the V3 amino acids of R5 tropic HIV-1 (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004). All five substitutions (E305K, R306S, R318T, R319G, and N320D) were accompanied by changes in electrical charge. As a result, the net charge of the V3 region shifted towards being more acidic (Fig. 1A). To determine whether this mutant, designated SHIV-MK1, was capable of replication within monkey cells, we spinoculated SHIV-MK1 on rhPBMCs at an MOI of 0.1. The RT activity in the supernatant was monitored daily. The X4 tropic SHIV-DH12R-CL-7 and parental SHIV-KS661 actively replicated on rhPBMCs, reaching its peak RT activity level 4 days after inoculation. The R5 tropic SIVmac239 reached its peak RT value at the same time point; however, the peak value was less than 50% of that of SHIV-DH12R-CL-7 and SHIV-KS661. SHIV-MK1 also replicated on rhPBMCs, but it took 2 days longer to reach peak RT activity levels, and the peak RT value was significantly lower than that of the parental SHIV-KS661 (Fig. 1B).

Next, to determine whether SHIV-MK1 was capable of utilizing CCR5, but not CXCR4, we conducted a small molecule inhibitor assay. Briefly, SIVmac239, SHIV-DH12R-CL-7, SHIV-KS661, or SHIV-MK1 was spinoculated on rhPBMCs that were preincubated with AD101 (R5 inhibitor), AMD3100 (X4 inhibitor), or both inhibitors at various concentrations. The supernatant RT activities were measured 5 days post-inoculation. The replication of X4 tropic SHIV-DH12-CL-7 was inhibited with AMD3100 in a dose-dependent manner; however, it was not restrained with AD101 as described previously (Igarashi et al., 1999, 2003; Sadjadpour et al., 2004). The same pattern was observed in SHIV-KS661-infected rhPBMCs, thus indicating that this virus is also an X4 tropic virus. In contrast, there was no replication inhibition of R5 tropic SIVmac239 in the presence of AMD3100; however, dose-dependent inhibition was observed in the presence of AD101. This result is consistent with other reports (Marcon et al., 1997; Zhang et al., 2000). SHIV-MK1 exhibited the same inhibition profile as SIVmac239, indicating that this virus predominantly utilizes CCR5, but not CXCR4, as an entry secondary receptor.

R5 tropic SHIV-MK1 can replicate in rhesus macaques

To determine whether SHIV-MK1 is capable of replication in rhesus macaques, we intravenously inoculated two monkeys (MM482 and MM483) with 20,000 TCID50 SHIV-MK1. Large amount of virus was inoculated to this group of monkey because *in vitro* replication of SHIV-MK1 was significantly weak compared with that of parental SHIV-KS661. As a control, two other monkeys (MM455 and MM459) were infected with 2000 TCID50 SHIV-KS661, a sufficient amount of virus to induce AIDS-like symptoms (Fukazawa et al., 2008; Miyake et al., 2006). Plasma viral RNA loads were monitored periodically using quantitative RT-PCR. Both groups of infected monkeys exhibited viremia, which reached peak plasma viral RNA loads of 10^6–10^8 copies/ml 2 weeks post-infection. In SHIV-KS661-infected monkeys, the set point of plasma viral RNA loads was between 10^4 and 10^6 copies/ml (Fig. 2Ai). In contrast, the plasma viral RNA load in one of the two monkeys infected with SHIV-MK1 was undetectable by 6 weeks post-infection, although 10-fold more virus was inoculated. The other monkey maintained 10^3–10^4 copies/ml plasma viral RNA for more than 25 weeks post-infection (Fig. 2Aii).

Next, circulating CD4+ T lymphocytes were analyzed by fluorescence activated cell sorting (FACS) to elucidate the impact of infection on lymphocyte subsets. As previously reported, X4 tropic SHIV-KS661 caused a massive depletion of circulating CD4+ T lymphocytes within 4 weeks post-infection (Fig. 2Bi). In contrast, circulating CD4+ T lymphocytes transiently decreased in monkeys infected with SHIV-MK1; however, they tended to recover by 24 weeks post-infection (Fig. 2Bii).

Because X4 tropic viruses preferably target naive CD4+ T lymphocytes, and R5 tropic viruses preferably target memory CD4+ T lymphocytes, circulating memory and naive CD4+ T lymphocytes were analyzed. The ratios of memory and naive CD4+ T cells were monitored 0, 2, 4, and 8 weeks post-SHIV-MK1 infection (Fig. 2C). Consistent with previous reports (Nishimura et al., 2004), X4 tropic SHIV-KS661 preferentially depleted naive T lymphocytes by 2 weeks post-infection. Although there was a subtle reduction in CD4+ T lymphocytes, the ratio of memory and naive CD4+ T lymphocytes did not change in SHIV-MK1-infected monkeys. This result indicates that a reduction in CD4+ T cells during SHIV-MK1 infection was not sufficient to alter the ratio of memory T cells, at least in circulating T lymphocytes.

The intestine is an effecter site where most CD4+ T lymphocytes are memory cells, and is the primary target for R5 tropic viruses (Harouse et al., 1999; Veazey et al., 1998). To elucidate the impact of viral infection in the intestine, tissue samples from the jejunum were obtained periodically and CD4+ T lymphocyte subsets were analyzed (Fig. 2D). As reported previously, CD4+ T lymphocytes of KS661-infected monkeys were depleted by 4 weeks post-infection (Fukazawa et al., 2008; Miyake et al., 2006). Although CD4+ T lymphocyte depletion was observed in one of the SHIV-MK1-infected monkeys (MM482) within 4 weeks post-infection, CD4+ T lymphocytes recovered as plasma viral RNA loads decreased. Another SHIV-MK1 infected monkey (MM483) whose plasma viral RNA load dropped below detectable levels showed only a transient reduction in CD4+ lymphocytes 5 weeks after infection. Taken together, these results suggest that, although the magnitude of jejunal CD4+ T-cell reduction was greater than that of circulating CD4+ T cells, the capability of SHIV-MK1 to cause CD4+ T lymphocyte depletion in the jejunum is not as strong as the parental SHIV-KS661.