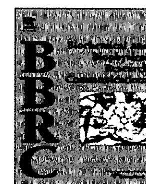
Biochemical and Biophysical Research Communications 408 (2011) 615–619

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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



Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

Yusuke Takahara [a,b], Saori Matsuoka [b], Tetsuya Kuwano [a], Tetsuo Tsukamoto [a], Hiroyuki Yamamoto [b], Hiroshi Ishii [a,b], Tadashi Nakasone [b], Akiko Takeda [b], Makoto Inoue [c], Akihiro Iida [c], Hiroto Hara [c], Tsugumine Shu [c], Mamoru Hasegawa [c], Hiromi Sakawaki [d], Mariko Horiike [d], Tomoyuki Miura [d], Tatsuhiko Igarashi [d], Taeko K. Naruse [e], Akinori Kimura [e], Tetsuro Matano [a,b,*]

[a] *Division for AIDS Vaccine Development, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*
[b] *AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[c] *DNAVEC Corporation, 6 Ohkubo, Tsukuba, Ibaraki 300-2611, Japan*
[d] *Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[e] *Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan*

ARTICLE INFO

Article history:
Received 12 April 2011
Available online 21 April 2011

Keywords:
AIDS vaccine
HIV
SIV
CTL
Immunodominance

ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

* Corresponding author at: AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Fax: +81 3 5285 1165.
E-mail address: tmatano@nih.go.jp (T. Matano).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.04.071

$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian-human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

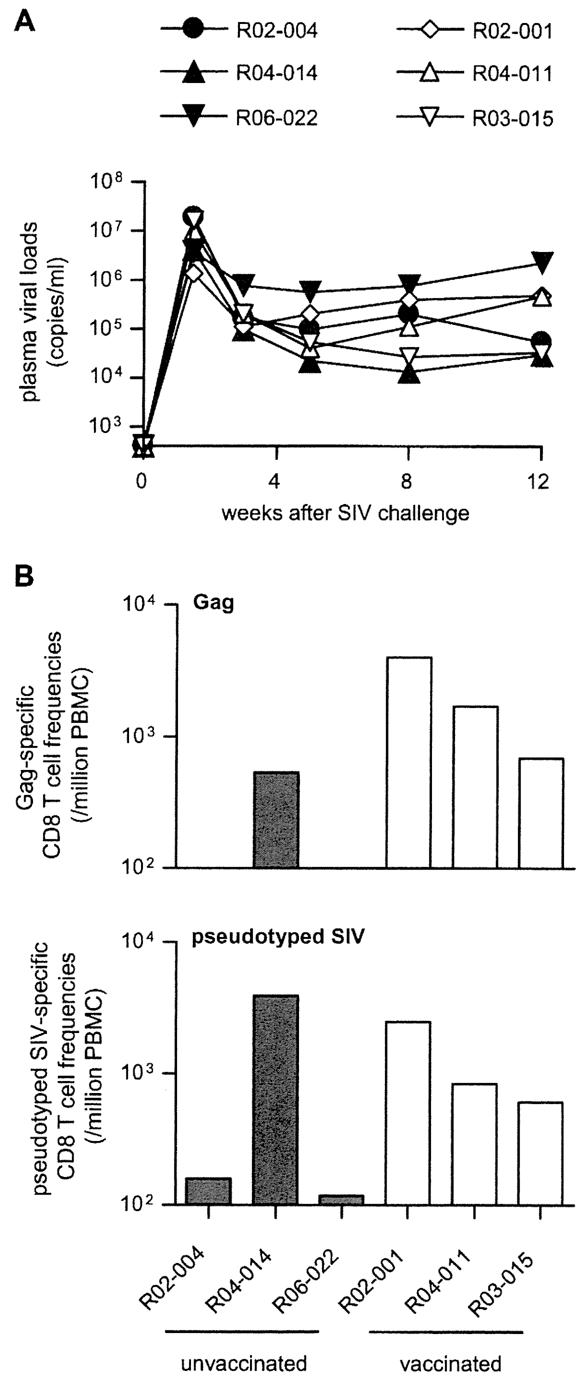


Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific CD8+ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific CD8+ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

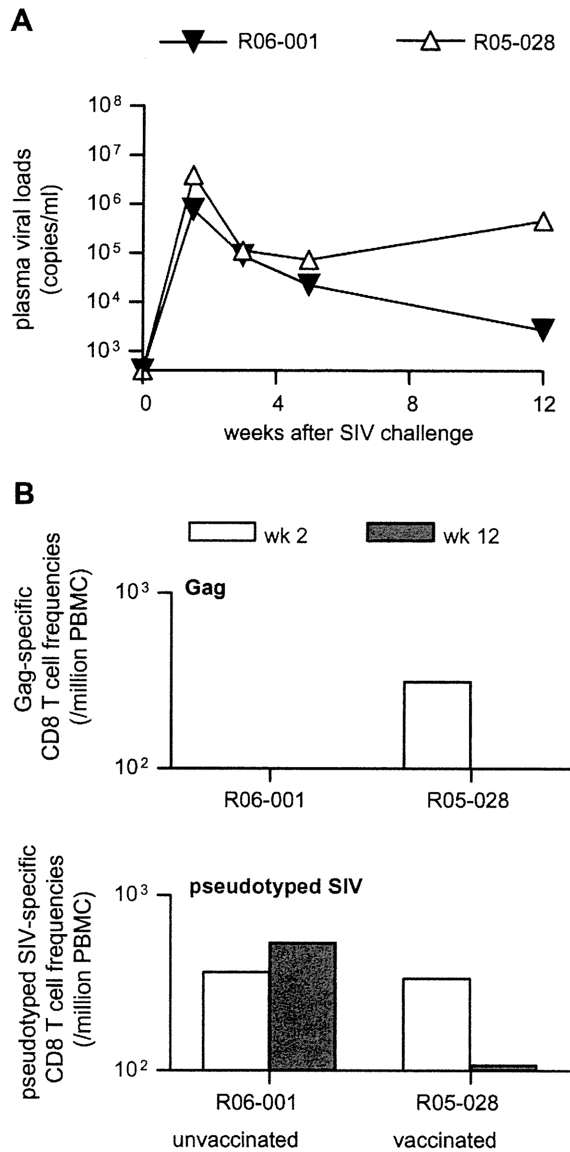


Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06-001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific CD8$^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

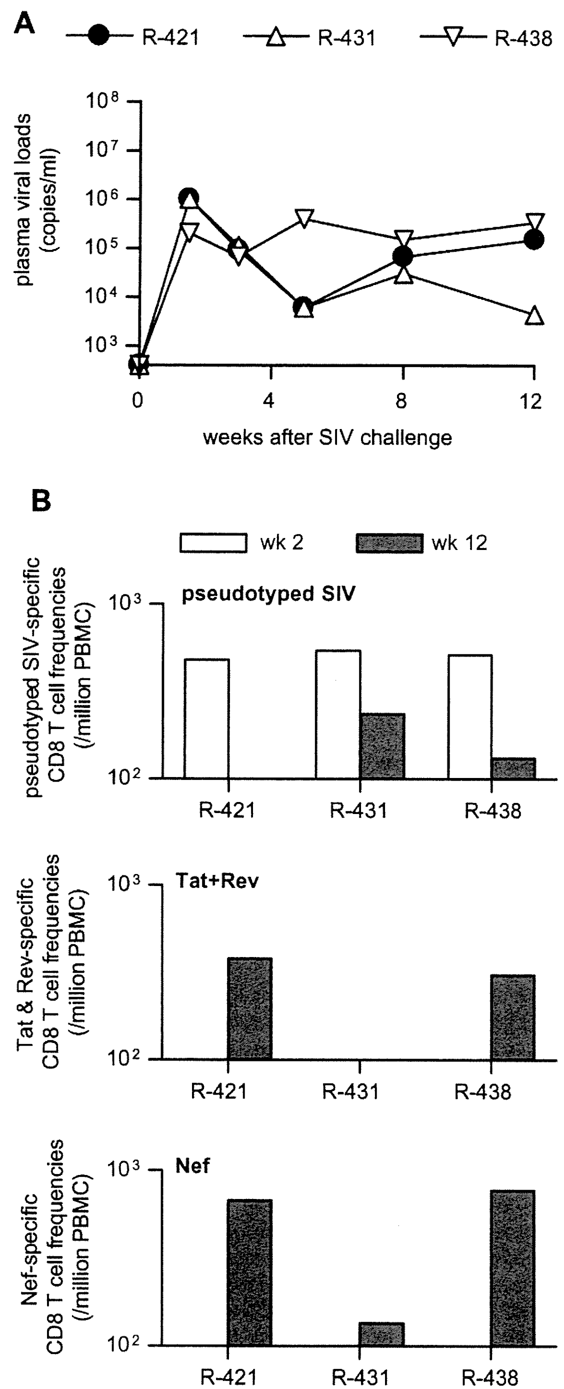


Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific CD8$^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific CD8$^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ$^+$ CD8$^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R- 421					++											
R- 431					+											
R- 438	++		+							++						
wk 12																
R- 421		++			++				+		+	+	+			++
R- 431					+		+			++						
R- 438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

This work was supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, Grants-in-aid from the Ministry of Health, Labor, and Welfare, and a Grant from Takeda Science Foundation in Japan.

References

[1] R.A. Koup, J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, D.D. Ho, Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome, J. Virol. 68 (1994) 4650–4655.

[2] P. Borrow, H. Lewicki, B.H. Hahn, G.M. Shaw, M.B. Oldstone, Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection, J. Virol. 68 (1994) 6103–6110.

[3] T. Matano, R. Shibata, C. Siemon, M. Connors, H.C. Lane, M.A. Martin, Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques, J. Virol. 72 (1998) 164–169.

[4] X. Jin, D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, L.G. Kostrikis, L. Zhang, A.S. Perelson, D.D. Ho, Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques, J. Exp. Med. 189 (1999) 991–998.

[5] P.J. Goulder, D.I. Watkins, HIV and SIV CTL escape: implications for vaccine design, Nat. Rev. Immunol. 4 (2004) 630–640.

[6] T. Matano, M. Kano, H. Nakamura, A. Takeda, Y. Nagai, Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen, J. Virol. 75 (2001) 11891–11896.

[7] T. Matano, M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D.H. O'Connor, D.I. Watkins, Y. Nagai, Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial, J. Exp. Med. 199 (2004) 1709–1718.

[8] Y. Takahashi-Tanaka, M. Yasunami, T. Naruse, K. Hinohara, T. Matano, K. Mori, M. Miysazawa, M. Honda, Y. Yasutomi, Y. Nagai, A. Kimura, Reference strand-mediated conformation analysis (RSCA)-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci, Electrophoresis 28 (2007) 918–924.

[9] M. Kawada, T. Tsukamoto, H. Yamamoto, N. Iwamoto, K. Kurihara, A. Takeda, C. Moriya, H. Takeuchi, H. Akari, T. Matano, Gag-specific cytotoxic T lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial, J. Virol. 82 (2008) 10199–10206.

[10] T. Tsukamoto, A. Takeda, T. Yamamoto, H. Yamamoto, M. Kawada, T. Matano, Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4+ T-Cell help on control of a simian immunodeficiency virus challenge in rhesus macaques, J. Virol. 83 (2009) 9339–9346.

[11] H. Yamamoto, M. Kawada, A. Takeda, H. Igarashi, T. Matano, Post-infection immunodeficiency virus control by neutralizing antibodies, PLoS ONE 2 (2007) e540.

[12] R. Shibata, F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, M.A. Martin, Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing, J. Infect. Dis. 176 (1997) 362–373.

[13] H.O. Li, Y.F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y.S. Lee, M. Fukumura, A. Iida, A. Kato, Y. Nagai, M. Hasegawa, A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression, J. Virol. 74 (2000) 6564–6569.

[14] A. Takeda, H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, T. Matano, Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model, J. Virol. 77 (2003) 9710–9715.

[15] H.W. Kestler 3rd, D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, R.C. Desrosiers, Importance of the nef gene for maintenance of high virus loads and for development of AIDS, Cell 65 (1991) 651–662.

[16] T. Matano, M. Kano, T. Odawara, H. Nakamura, A. Takeda, K. Mori, T. Sato, Y. Nagai, Induction of protective immunity against pathogenic simian immunodeficiency virus by a foreign receptor-dependent replication of an engineered avirulent virus, Vaccine 18 (2000) 3310–3318.

[17] L.M. Albritton, L. Tweng, D. Scadden, J.M. Cunningham, A putative murine retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection, Cell 57 (1989) 659–666.

[18] M. Kawada, T. Tsukamoto, H. Yamamoto, A. Takeda, H. Igarashi, D.I. Watkins, T. Matano, Long-term control of simian immunodeficiency virus replication with central memory CD4+ T-cell preservation after nonsterile protection by a cytotoxic T-lymphocyte-based vaccine, J. Virol. 81 (2007) 5202–5211.

[19] N. Iwamoto, T. Tsukamoto, M. Kawada, A. Takeda, H. Yamamoto, H. Takeuchi, T. Matano, Broadening of CD8+ cell responses in vaccine-based simian immunodeficiency virus controllers, AIDS 24 (2010) 2777–2787.

[20] S. Tenzer, E. Wee, A. Burgevin, G. Stewart-Jones, L. Friis, K. Lamberth, C.H. Chang, M. Harndahl, M. Weimershaus, J. Gerstoft, N. Akkad, P. Klenerman, L. Fugger, E.Y. Jones, A.J. McMichael, S. Buus, H. Schild, P. van Endert, A.K. Iversen, Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance, Nat. Immunol. 10 (2009) 636–646.

[21] P.J.R. Goulder, D.I. Watkins, Impact of MHC class I diversity on immune control of immunodeficiency virus replication, Nat. Rev. Immunol. 8 (2008) 619–630.

[22] H. Streeck, J.S. Jolin, Y. Qi, B. Yassine-Diab, R.C. Johnson, D.S. Kwon, M.M. Addo, C. Brumme, J.P. Routy, S. Little, H.K. Jessen, A.D. Kelleher, F.M. Hecht, R.P. Sekaly, E.S. Rosenberg, B.D. Walker, M. Carrington, M. Altfeld, Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells, J. Virol. 83 (2009) 7641–7648.

[23] L. Alexander, L. Denekamp, S. Czajak, R.C. Desrosiers, Suboptimal nucleotides in the infectious, pathogenic simian immunodeficiency virus clone SIVmac239, J. Virol. 75 (2001) 4019–4022.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 27, Number 5, 2011
© Mary Ann Liebert, Inc.
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-*SpeI* (5′-AGG TTT ACT AGT ACC ACC ACA TGT TTT TAT CTC-3′) for Ig heavy chain, VK1a-*SacI* (5′-GAC ATC GAG CTC ACC CAG TCT CCA-3′) and CK-*XbaI* (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-*SacI* (5′-GGG CCC AGG CGG CCG AGC TCG TGC TGA CGC AGC CTC CCT C-3′) and CL2-*XbaI* (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 *SfiI*. 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-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′)

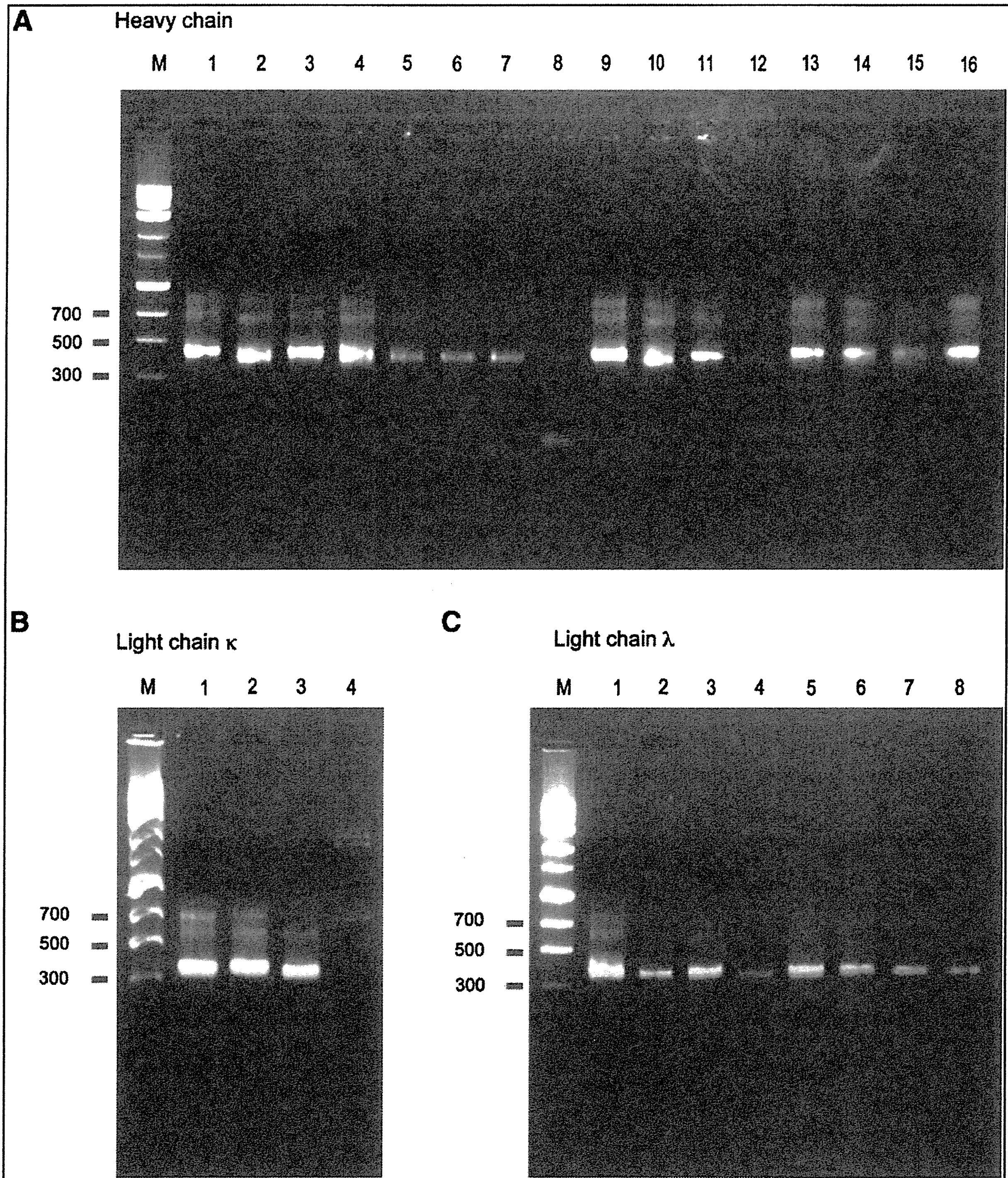


FIG. 1. Ig heavy chain and light chain κ and λ variable regions were successfully amplified by PCR using primers shown in Table 1. **(A)** The Ig heavy chain variable region was amplified using the following primer pairs: lane 1, RhFabVH17-F and RhFabVHJ1-B; 2, RhFabVH17-F and RhFabVHJ2-B; 3, RhFabVH17-F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

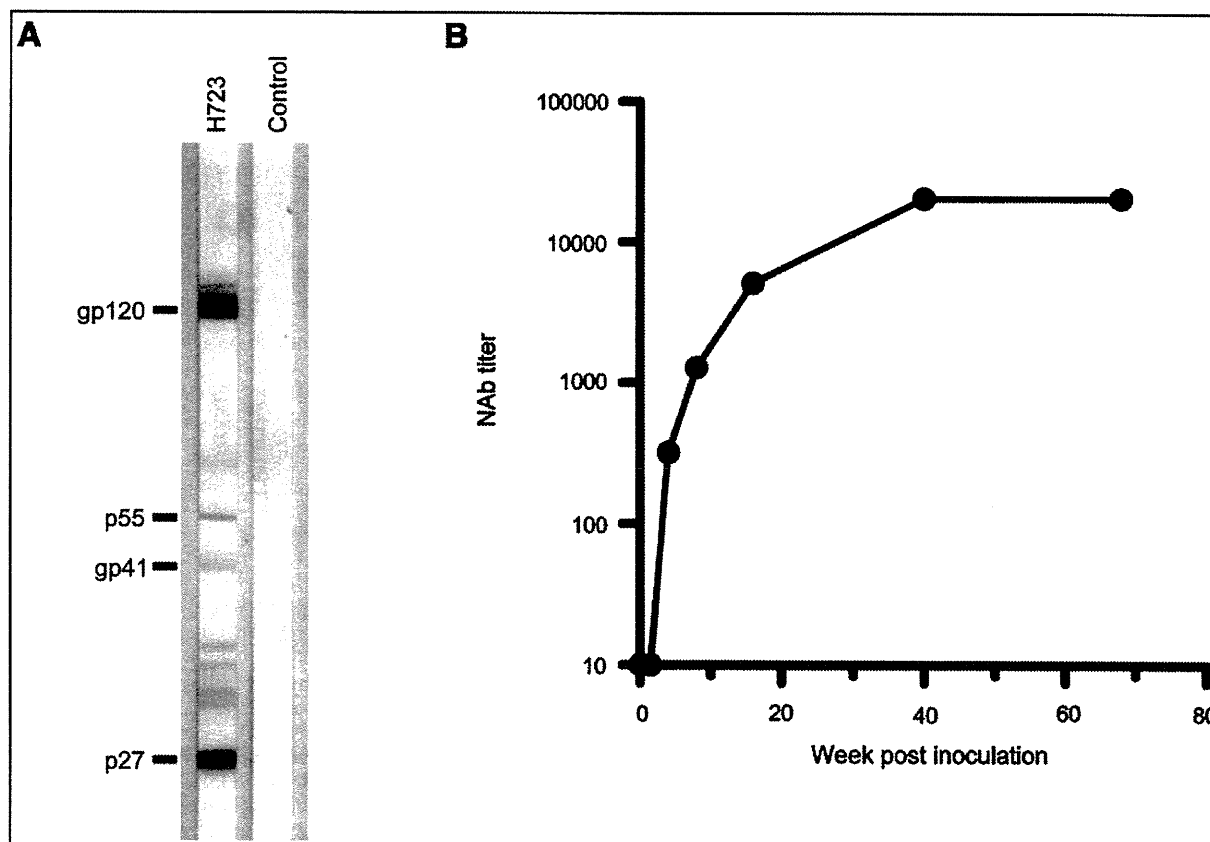


FIG. 2. Antibody response in SIVsmH635FC-infected macaque H723. **(A)** Western blot analysis showed that antibodies in plasma from H723 at death recognized Env gp120 and gp41 and Gag p55 and p27. **(B)** H723 maintained a high NAb titer during the course of infection. Neutralizing titer was determined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

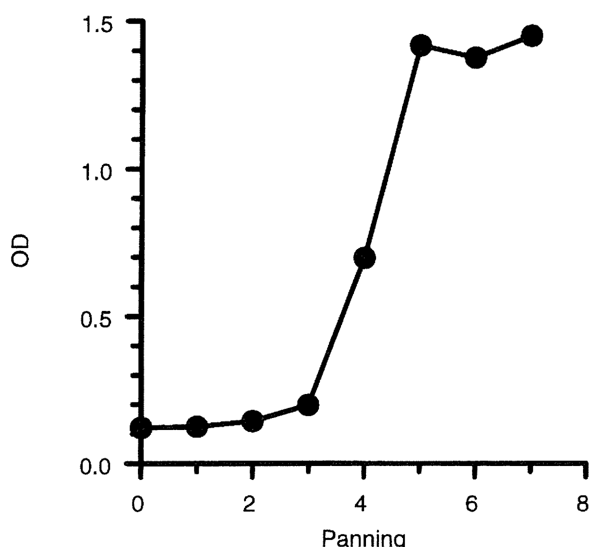


FIG. 3. Reactivity of phage to SIV Ag increased from the fourth round of panning. Phage samples that were eluted after panning were examined by ELISA for their ability to bind SIV proteins.

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.

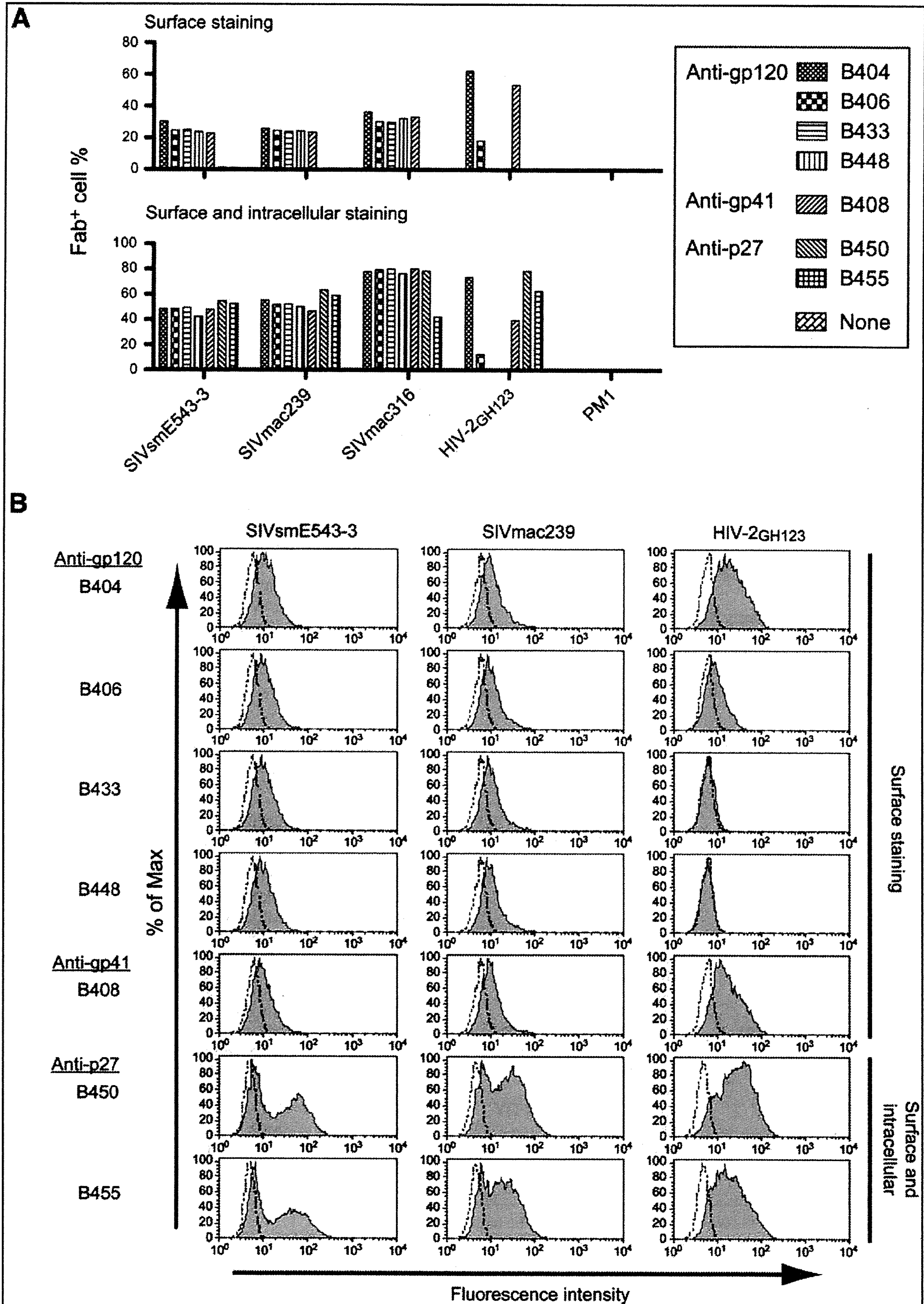
Selection of SIV-specific Fab clones by panning

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

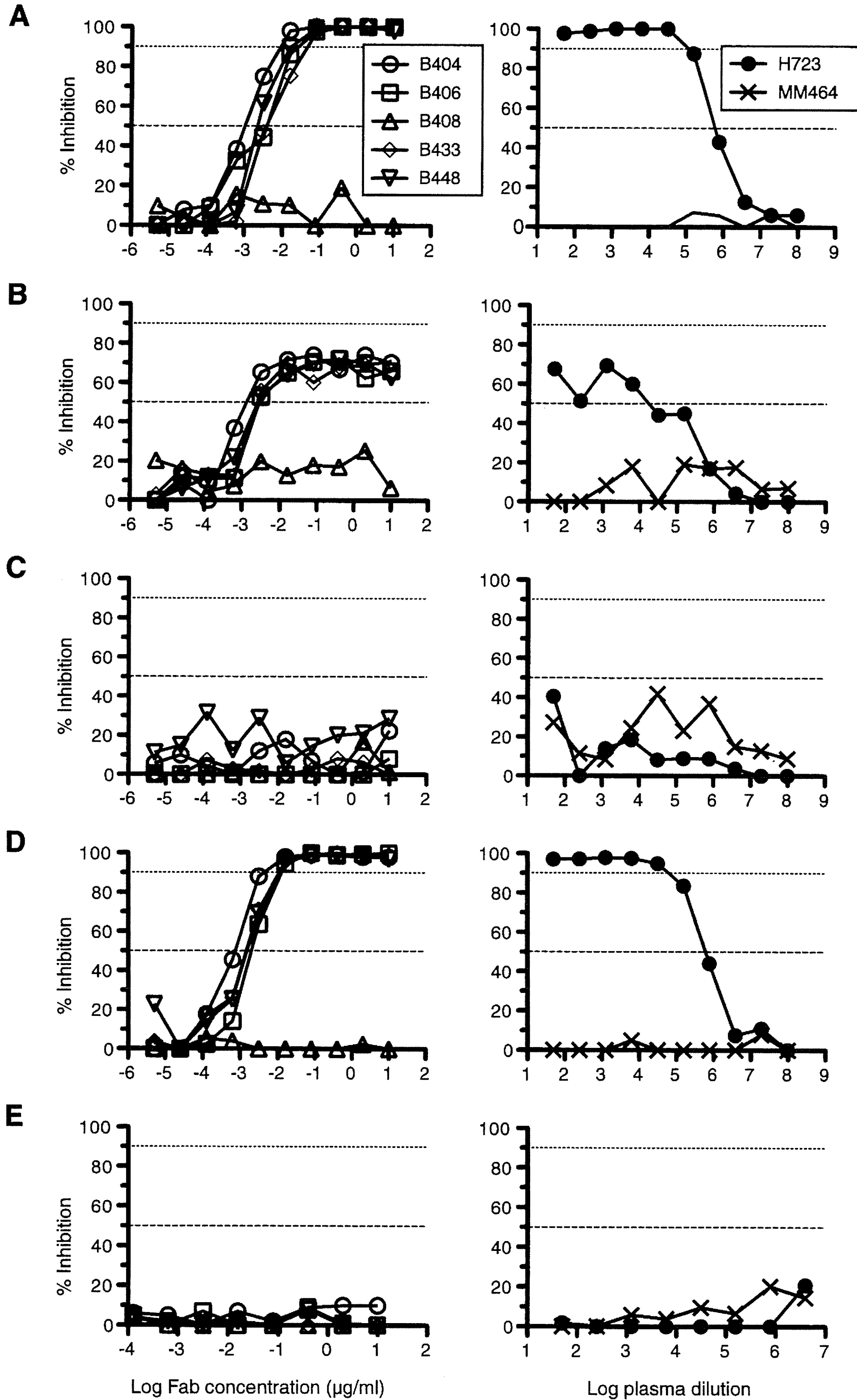
SIVmac239, another heterologous strain, and HIV-2_{GH123} were not neutralized by any Fab (Fig. 5C and E). HIV-2_{GH123} was not neutralized, perhaps because of its low cross-reactivity, though B404 bound HIV-2_{GH123} (Fig. 4). Unsuccessful neutralization of SIVmac239 may be related to the Env structure,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

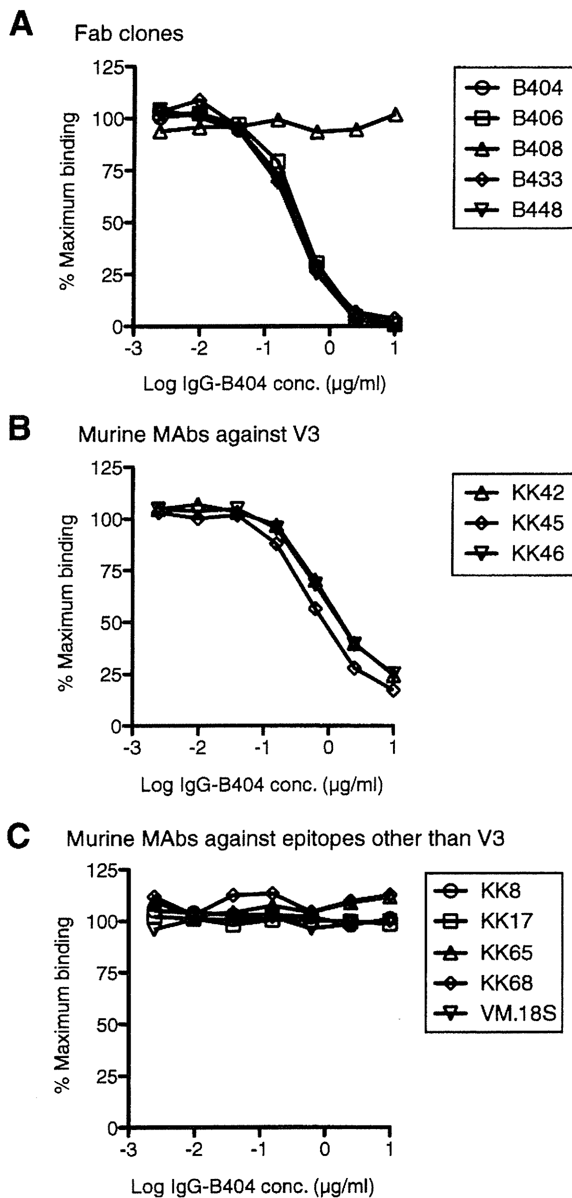


FIG. 6. Identification of epitope specificity of anti-gp120 Fab clones by competition ELISA. **(A)** IgG-B404 inhibited the binding of anti-gp120 Fabs, B404, B406, B433, and B448, but did not inhibit anti-gp41 Fab B406. **(B)** IgG-B404 competed with the binding of murine MAbs, KK42, KK45, and KK46,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

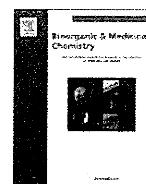
E-mail: tkuwata@kumamoto-u.ac.jp

Bioorganic & Medicinal Chemistry 19 (2011) 6735–6742

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

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



Small molecular CD4 mimics as HIV entry inhibitors

Tetsuo Narumi [a], Hiroshi Arai [a], Kazuhisa Yoshimura [b], Shigeyoshi Harada [b], Wataru Nomura [a], Shuzo Matsushita [b], Hirokazu Tamamura [a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan

ARTICLE INFO

Article history:
Received 5 August 2011
Revised 23 September 2011
Accepted 24 September 2011
Available online 29 September 2011

Keywords:
CD4 mimic
HIV entry
gp120-CD4 interaction
Phe43 cavity

ABSTRACT

Derivatives of CD4 mimics were designed and synthesized to interact with the conserved residues of the Phe43 cavity in gp120 to investigate their anti-HIV activity, cytotoxicity, and CD4 mimicry effects on conformational changes of gp120. Significant potency gains were made by installation of bulky hydrophobic groups into the piperidine moiety, resulting in discovery of a potent compound with a higher selective index and CD4 mimicry. The current study identified a novel lead compound **11** with significant anti-HIV activity and lower cytotoxicity than those of known CD4 mimics.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

The dynamic supramolecular mechanism of HIV cellular invasion has emerged as a key target for blocking HIV entry into host cells.[1] HIV entry begins with the interaction of a viral envelope glycoprotein gp120 and a cell surface protein CD4.[2] This triggers extensive conformational changes in gp120 exposing co-receptor binding domains and allowing the subsequent binding of gp120 to a co-receptor, CCR5[3]/CXCR4.[4] Following the viral attachment and co-receptor binding, gp41, another viral envelope glycoprotein mediates the fusion of the viral and cell membranes, thus completing the infection. Molecules interacting with each of these steps are potential candidates for anti-HIV-1 drugs. In particular, discovery and development of novel drugs that inhibit the viral attachment are required for blocking the HIV infection at an early stage.[5]

In 2005, small molecular CD4 mimics targeting the viral attachment were identified by an HIV syncytium formation assay and shown to bind within the Phe43 cavity, a highly conserved pocket on gp120,[6] which is a hydrophobic cavity occupied by the aromatic ring of Phe43 of CD4.[7] These molecules are comprised of three essential moieties: an aromatic ring, an oxalamide linker, and a piperidine ring (Fig. 1) and show micromolar order potency against diverse HIV-1 strains including laboratory and primary isolates. Furthermore, they possess the unique ability to induce the conformational changes in gp120 required for binding with soluble CD4.[8] Such CD4 mimicry can be an advantage for rendering the envelope more sensitive to neutralizing antibodies.[9] While such properties are promising for the development of HIV entry inhibitors and the use combinatorially with neutralizing antibodies, cytotoxicity is one of the drawbacks of CD4 mimics.

To date, we and others have performed structure–activity relationship (SAR) studies of CD4 mimics based on modifications of the aromatic ring, the oxalamide linker, and the piperidine moiety of CD4 mimics. In an initial survey of SAR studies of NBD-556 and NBD-557, Madani et al. revealed that potency (i.e., CD4 binding and mimicry) was highly sensitive to modifications of the aromatic ring, which is thought to bind in the Phe43 cavity of gp120 (Fig. 1). The CD4 mimic analogs (JRC-II-191) with a *para*-chloro-*meta*-fluorophenyl ring had significantly increased affinity for gp120.[10] Our SAR studies also revealed that a certain size and electron-withdrawing ability of the *para*-substituents are indispensable for potent anti-HIV activity.[11] Furthermore, the replacement of the chlorine group at the *para* position with a methyl group which is almost as bulky as a bromine atom leads to improvement of solubility of the compounds in buffer to provide the reproducibility in the biological studies with comparable biological activities.

Further SAR studies were focused on the piperidine moiety of CD4 mimics to investigate its contribution to biological activities, and we found that the piperidine ring is critical for the CD4 mimicry on the conformational changes in gp120 and that substituents on the nitrogen of the piperidine moiety can contribute significantly to both anti-HIV activity and cytotoxicity.[12] Based on these SARs and our modeling study, we speculate that interactions of the piperidine moiety with several amino acids in the vicinity of the Phe43 cavity in gp120, specifically an electrostatic interaction with

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.
E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.09.045