the two MAbs 2F5 and 2G12 or a triple combination of HIVIG, 2F5, and 2G12, as previously reported (41, 43), we postulated that KD-247 was sufficiently potent to achieve protection of monkeys against a pathogenic SHIV challenge. Since our previous experience (9) has taught us to expect approximately 500 to 1,000 μg/ml in sera from monkeys passively immunized with 30 to 45 mg of antibody per kg of body weight, the potency of KD-247 should prove sufficient for passive transfer experiments of effective antibodies in animals in vivo. We also expected that a single passive transfer of KD-247 via inoculation with 15 and 30 mg of antibody would result in approximately 150 to 500 μg/ml of plasma concentration at the time of viral challenge. As expected, we found an AUC value of 1.8 to 5.0 mg · day/ml. Consequently, we found that animals passively immunized with 45 mg/kg of KD-247 showed 678 and 866 μg/ml of KD-247 in plasma at the time of viral challenge and an AUC value of 5.6 and 6.5 mg · day/ml. Those animals were provided sterile protection against intravenous challenge with the pathogenic virus SHIV C2/1. The protective endpoint titers of neutralization antibodies in plasma at the time of virus inoculation were 1:160 in both animals that elicited sterile immunity, and a high titer of neutralization activity in plasma was similarly detected in completely protected monkeys, as described previously by Nishimura et al. (26) and Parren et al. (29). Thus, the high titers of neutralization activity in plasma confer sterile protection against viral challenge in the passively immunized animals with neutralizing antibodies. Furthermore, the pharmacokinetic information consisting of the plasma concentration of the neutralizing antibodies at the time of viral challenge and the AUC value may be closely related to the ability of the antibody to provide sterile protection against viral challenge. Since those protected macaques demonstrated the inhibition of $CD4^+$ cell loss, the pharmacokinetic properties of KD-247 may also be closely associated with the inhibition of $CD4^+$ cell decline in the peripheral circulation of the challenged monkeys.

In this study, we also detected lower viremia with lesser $CD4^+$ cell decline in animals that were inoculated with intermediate doses of antibody. However, we noted that the lesser doses of the antibody provided complete protection against enhanced rates of the $CD4^+$ $CD95^+$ cell subpopulation in the peripheral circulation of the challenged animals, suggesting that the reshaping MAb might be able to control the activation of peripheral $CD4^+$ T cells in animals by its passive transfer. Although the number of monkeys enrolled in this study was limited, it remains noteworthy that a single inoculation with KD-247, even at a suboptimal dose for viral protection, appeared to be effective for maintaining $CD4^+$ T cells in monkeys inoculated with virus. Since it has been previously reported that the limited effect of neutralizing antibody may be related to the rapid appearance of an escape mutant in infected individuals, high titers of neutralization activity should be generated in the passively immunized animals (25, 33, 44). In our preliminary study, we isolated the escape mutant from the neutralization resistance virus HIV-1_{JR-FL} in the presence of KD-247: at passage 8 of the culture in the presence of 1,000 μg/ml KD-247, one amino acid substitution, GPGR to GPER, was identified in the V3 tip (K. Yoshimura et al., unpublished results). Collectively, these results suggest that KD-247 shows clinical promise both for passive immunization and as a strategy for preventing viral spread in phenotype-matched HIV-infected individuals.

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Vaccine-based, long-term, stable control of simian/ human immunodeficiency virus 89.6PD replication in rhesus macaques

Hiroyuki Yamamoto,[1,2] Miki Kawada,[1,2] Tetsuo Tsukamoto,[1,2] Akiko Takeda,[1,2] Hiroko Igarashi,[2] Masaaki Miyazawa,[3] Taeko Naruse,[4] Michio Yasunami,[4] Akinori Kimura[4] and Tetsuro Matano[1,2,5,6]

Correspondence
Tetsuro Matano
matano@m.u-tokyo.ac.jp

[1]International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

[2]Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

[3]Department of Immunology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan

[4]Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

[5]AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

[6]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

The X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P (or 89.6PD) causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system, whereas pathogenic R5-tropic simian immunodeficiency virus (SIV) infection, like HIV-1 infection in humans, results in chronic disease progression in macaques. Recent pre-clinical vaccine trials inducing cytotoxic T lymphocyte (CTL) responses have succeeded in controlling replication of the former but shown difficulty in control of the latter. Analysis of the immune responses involved in consistent control of SHIV would contribute to elucidation of the mechanism for consistent control of SIV replication. This study followed up rhesus macaques that showed vaccine-based control of primary SHIV89.6PD replication and found that all of these controllers maintained viraemia control for more than 2 years. SHIV89.6PD control was observed in vaccinees of diverse major histocompatibility complex (MHC) haplotypes and was maintained without rapid selection of CTL escape mutations, a sign of particular CTL pressure. Despite the vaccine regimen not targeting Env, all of the SHIV controllers showed efficient elicitation of *de novo* neutralizing antibodies by 6 weeks post-challenge. These results contrast with our previous observation of particular MHC-associated control of SIV replication without involvement of neutralizing antibodies and suggest that vaccine-based control of SHIV89.6PD replication can be stably maintained in the presence of multiple functional immune effectors.

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INTRODUCTION

The well-established importance of cytotoxic T lymphocyte (CTL) responses in the control of immunodeficiency virus replication has led the way to development of prophylactic AIDS vaccine regimens that augment virus-specific CTL responses (Borrow *et al.*, 1994; Koup *et al.*, 1994; Matano *et al.*, 1998; Ogg *et al.*, 1998; Jin *et al.*, 1999; Schmitz *et al.*, 1999; McMichael & Hanke, 2003; Goulder & Watkins, 2004). In a model of X4-tropic simian/human immunodeficiency virus (SHIV) 89.6P or 89.6PD infection (Reimann *et al.*, 1996; Lu *et al.*, 1998), which causes rapid CD4$^+$ T-cell depletion leading to an acute crash of the host immune system in macaques, several pre-clinical trials of prophylactic AIDS vaccines have successfully shown that efficient CTL induction results in control of virus replication and prevention of acute AIDS progression (Barouch *et al.*, 2000; Amara *et al.*, 2001; Matano *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002; Willey *et al.*, 2003). In contrast, most trials of such CTL-based vaccines have failed to show viraemia control in models of R5-tropic simian immunodeficiency virus (SIV) infection, which result in chronic

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disease progression in macaques as in human immunodeficiency virus type 1 (HIV-1) infection in humans (Feinberg & Moore, 2002; Horton *et al.*, 2002; Casimiro *et al.*, 2005). Comparison of vaccine effects on virus replication in the acute AIDS model of X4-tropic SHIV infection with those in the chronic model of R5-tropic SIV infection could contribute to the development of an effective prophylactic AIDS vaccine for control of persistent HIV-1 replication.

We have developed a prophylactic AIDS vaccine using a DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector boost system and have shown its potential for efficient induction of Gag-specific CTL responses in Burmese rhesus macaques (Kano *et al.*, 2002; Matano *et al.*, 2004). In preclinical trials in an acute AIDS model, all of the macaques vaccinated with the DNA-prime/SeV-Gag vector boost system controlled SHIV89.6PD replication after challenge (Matano *et al.*, 2001; Takeda *et al.*, 2003). Furthermore, a trial of the prophylactic DNA-prime/SeV-Gag boost vaccine showed control of SIVmac239 replication leading to undetectable set-point plasma viraemia in five out of eight vaccinees (referred to as SIV controllers), despite failure of virus control in the other three vaccinees (referred to as SIV non-controllers) (Matano *et al.*, 2004). All of the SIV controllers showed rapid selection of viral CTL escape mutations, and analysis of the rhesus major histocompatibility complex (MHC) suggested that SIV control was associated with particular MHC haplotypes such as *90-120-Ia* and 'elite' CTL responses specific for the MHC-restricted epitopes (Matano *et al.*, 2004). Follow up of these SIV controllers revealed that some lost this control with accumulation of multiple viral CTL escape mutations (Kawada *et al.*, 2006).

In this study, we followed up, for more than 2 years, rhesus macaques that showed vaccine-based control of SHIV89.6PD replication (referred to as SHIV controllers). Our results showed durable and stable virus control in the SHIV controllers, contrasting with our previous observation in SIV controllers.

METHODS

Animal experiments. Ten vaccinated macaques used in our previous SHIV89.6PD challenge experiments (Matano *et al.*, 2001; Takeda *et al.*, 2003) were analysed in this study. The animal list is shown in Table 1. All were Burmese rhesus macaques (*Macaca mulatta*) and were maintained in accordance with the Guidelines for Laboratory Animals of the National Institute of Infectious Diseases and National Institute of Biomedical Innovation.

The immunization and challenge protocols have been described previously (Matano *et al.*, 2001; Takeda *et al.*, 2003). Of the ten macaques in the SHIV89.6PD challenge experiment, three (R00-013, R00-015 and R00-017) received a single intranasal immunization with replication-competent SeV expressing SIVmac239 Gag (SeV-Gag) (Kato *et al.*, 1996; Kano *et al.*, 2002) before challenge. Two (R99-007 and R99-011) received four immunizations with FMSIV DNA followed by a single SeV-Gag booster. The FMSIV plasmid DNA used in this DNA vaccination protocol (DNAv1) was constructed from an $SHIV_{MD14YE}$ molecular clone DNA (Shibata *et al.*, 1997a) by replacing SHIV *env* with ecotropic Friend murine leukemia virus (FMLV) *env*

Table 1. Summary of the vaccinees challenged with SHIV89.6PD

Animal	MHC I haplotype*	Vaccine protocol	Set-point virus load†	Virus load around year 2‡	Gag mutations around month 2§	Gag mutations after month 6‖
R00-013	ND	SeV-Gag	10^4–10^6	10^4–10^6 (at wk 52)	ND	ND
R00-015	*90-120-Ib*	SeV-Gag	<400	<400	None	None at wk 60
R00-017	*90-030-Ih*	SeV-Gag	<400	<400	None	None at wk 58
R99-007	ND	DNAv1/SeV-Gag	<400	<400 (at wk 28)	None	ND
R99-011	*90-010-Ie*	DNAv1/SeV-Gag	<400	<400	None	None at wk 51
R99-005	*90-010-Ie*	DNAv2/SeV-Gag	<400	<400	None	None at wk 49
R99-012	*90-030-Ih*	DNAv2/SeV-Gag	<400	<400	None	None at wk 51
R00-020	*90-122-Ie*	DNAv3/F$^-$SeV-Gag	<400	<400	None	None at wk 52
R00-023	ND	DNAv3/F$^-$SeV-Gag	<400	<400	None	None at wk 52
R00-024	*90-120-Ib*	DNAv3/F$^-$SeV-Gag	<400	<400	None	None at wk 52

*MHC I haplotype was determined by reference strand-mediated conformation analysis, as described previously (Arguello *et al.*, 1998; Matano *et al.*, 2004). MHC I haplotype *90-120-Ib* is derived from breeder R90-120, *90-010-Ie* from R90-010, *90-122-Ie* from R90-122 and *90-030-Ih* from R90-030. MHC I haplotypes *90-010-Ie* and *90-122-Ie* are identical.

†Plasma viral loads [RNA copies (ml plasma)$^{-1}$] around week 20.

‡Macaque R00-013 developed AIDS and was euthanized at week 53. Macaque R99-007 was euthanized at week 29 because of the limitation of available cage numbers.

§A *gag* gene fragment was amplified from plasma RNA at week 5 or from PBMC-derived DNA at week 7 or 8 and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes. The results are shown in Table 2.

‖A *gag* gene fragment was amplified from PBMC-derived DNA and subjected to sequencing to determine predominant mutations leading to Gag amino acid changes.

ND, Not determined.

(Matano *et al.*, 2000). Two macaques (R99-005 and R99-012) received four immunizations with both the FMSIV DNA and an FMLV receptor (mCAT1)-expression plasmid DNA (Albritton *et al.*, 1989) followed by a single SeV-Gag booster. This second DNA vaccination protocol (DNAv2) has been shown to elicit efficient CTL responses by confined mCAT1-dependent FMSIV replication (Matano *et al.*, 2000). Three macaques (R00-020, R00-023 and R00-024) received a single immunization with CMV-SHIVdEN DNA (DNAv3) followed by a single boost with an F-deleted replication-defective SeV-Gag (F^-SeV-Gag) (Li *et al.*, 2000; Takeda *et al.*, 2003). This CMV-SHIVdEN plasmid DNA was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA and had the genes encoding SIVmac239 Gag, Pol, Vif and Vpx, SIVmac239/HIV-1_{DH12} chimeric Vpr and HIV-1_{DH12} Tat and Rev (Matano *et al.*, 2004). All ten animals were challenged intravenously with 10 $TCID_{50}$ SHIV89.6PD (Lu *et al.*, 1998) approximately 3 months after the last immunization. Four unvaccinated animals were also challenged with SHIV89.6PD and all failed to control virus replication.

Quantification of plasma viral loads. Plasma RNA was extracted using a High Pure Viral RNA kit (Roche Diagnostics). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers to determine the end point. Plasma SIV RNA levels were calculated according to the Reed–Muench method, as described previously (Matano *et al.*, 2004). The lower limit of detection was approximately 4×10^2 RNA copies ml^{-1}.

Sequencing of viral and proviral genomes. Plasma RNA was extracted as described above and genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using a DNeasy kit (Qiagen). A fragment corresponding to nt 458–2185 (containing the entire *gag* region) in the SHIV89.6P genome (GenBank accession no. U89134) was amplified from plasma RNA by nested RT-PCR. Alternatively, fragments corresponding to nt 458–2185, 2019–3187, 3038–4197, 4056–5213, 5079–6250, 6065–7225, 7047–8176 and 7998–9172 in the SHIV89.6P genome were amplified from proviral DNA by nested PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, PCR products were subcloned into plasmids using a TOPO cloning system (Invitrogen) and sequenced.

Measurement of virus-specific T-cell levels by intracellular cytokine staining. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (Matano *et al.*, 2001, 2004). In brief, PBMCs were co-cultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) infected with a vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 for SHIV-specific stimulation. The pseudotyped virus was obtained by co-transfection of COS-1 cells with a VSV-G-expression plasmid and the SIVGP1 DNA, an *env*- and *nef*-deleted SHIV molecular clone DNA, constructed by removing the whole FMLV *env* region from the FMSIV DNA. Alternatively, PBMCs were co-cultured with B-LCLs pulsed with peptide mixture (final concentration of each peptide, 0.5–2 μM) for peptide-specific stimulation. A panel of 117 overlapping peptides (15–17 aa in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag sequence (Sigma-Aldrich) were divided into ten pools (1–10) each consisting of 11 or 12 peptides. Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. 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 antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ^+ T-cell frequencies from those after SHIV-specific or peptide-specific stimulation. Specific T-cell levels of < 100 cells per 10^6 PBMCs were considered negative.

Measurement of virus-specific neutralizing titres. We performed a neutralizing assay for the measurement of virus-specific neutralizing titres in plasma, as described previously (Shibata *et al.*, 1997b). Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ SHIV89.6PD. In each mixture, 5 μl diluted plasma was incubated with 5 μl virus. After a 45 min incubation at room temperature, each 10 μl mixture was added to 5×10^4 MT4 cells in a well of a 96-well plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by ELISA for detection of SIV p27 core antigen (Beckman Coulter) to determine the 100 % neutralizing end point. The lower limit of detection was a titre of 1 : 2.

RESULTS

MHC haplotypes of the SHIV controllers

In our previous SHIV89.6PD challenge experiment (Matano *et al.*, 2001; Takeda *et al.*, 2003), three animals received a single SeV-Gag vaccination alone, whilst the remaining seven animals were immunized with a DNA-prime/SeV-Gag boost vaccine before challenge (Table 1). The seven animals vaccinated with the prime–boost vaccine (R99-007, R99-011, R99-005, R99-012, R00-020, R00-023 and R00-024) were able to control virus replication, with undetectable set-point plasma viraemia. Two (R00-015 and R00-017) of the three animals vaccinated with SeV-Gag alone were also able to control viraemia, but the remaining one (R00-013) failed to control virus replication and showed acute CD4$^+$ T-cell depletion. This animal R00-013 developed AIDS and was euthanized at week 53.

In the present study, we determined the MHC class I (MHC I) haplotypes of the SHIV controllers and their viral genome sequences at around 1 or 2 months after challenge to examine whether SHIV controllers showed rapid selection of CTL escape mutations as observed in our previous analysis, in particular MHC-associated control of SIV replication. Importantly, control of SHIV89.6PD replication was observed in vaccinees with diverse MHC haplotypes (Table 1). Analysis of the proviral *gag* region in PBMCs at around week 8 showed a predominance of the wild-type sequence in all nine SHIV controllers (Table 2). Sequencing of the plasma viral *gag* region at week 5 in three of them confirmed the lack of dominant mutations (Table 2). Thus, the SHIV controllers controlled virus replication without rapid selection of CTL escape mutations.

Follow-up of the SHIV controllers

We followed up eight of the nine SHIV controllers except for one animal, R99-007, which was euthanized at week 29 because of a limitation on available cage numbers (Table 1). All eight SHIV controllers maintained control of virus replication for more than 2 years (Fig. 1). Viraemia was undetectable and peripheral CD4$^+$ T-cell counts were maintained during the observation period. Analysis of the *gag* region in PBMC-derived proviral DNA revealed that the wild-type sequence was still dominant around 1 year after challenge in all eight (Table 1). Additionally, we succeeded

Table 2. Mutations in SHIV *gag* at 1 or 2 months post-challenge

A *gag* gene fragment was amplified by nested PCR from PBMC-derived DNA at week 7 (in R00-017) or week 8 (in others) or by nested RT-PCR from plasma RNA at week 5. The viral *gag* fragment was amplified from plasma RNA in only three of the nine SHIV controllers (R00-017, R00-023 and R00-024); this was due to lower viral loads at week 5 in the remaining SHIV controllers.

Animal	Frequency*	Position of Gag changes (aa)†
Proviral DNA at week 7 or 8		
–R00-015	8/9	None
	1/9	373
–R00-017	9/10	None
	1/10	384
–R99-007	8/9	None
	1/9	485
–R99-011	9/10	None
	1/10	141
–R99-005	8/9	None
	1/9	495
–R99-012	8/10	None
	1/10	210
	1/10	372, 456
–R00-020	9/9	None
–R00-023	7/10	None
	3/10	385
–R00-024	7/7	None
Plasma RNA at week 5		
–R00-017	2/9	None
	1/9	49
	1/9	208
	1/9	443
	1/9	49, 103
	1/9	270, 448
	1/9	59, 232, 293
	1/9	391, 481, 499
–R00-023	2/11	None
	2/11	218
	1/11	27
	1/11	434
	1/11	444, 493
	1/11	76, 182, 379
	1/11	118, 272, 380
	1/11	5, 140, 312, 434
	1/11	6, 17, 112, 205
–R00-024	2/9	None
	1/9	227
	1/9	42, 301
	1/9	272, 434
	1/9	9, 48, 367
	1/9	50, 176, 247
	1/9	103, 364, 386
	1/9	108, 137, 364, 386, 411

*Number of clones with change(s)/total number of clones.
†Amplified *gag* fragments were subcloned into plasmids for sequencing and the positions of amino acid changes in SHIV Gag in each clone are shown.

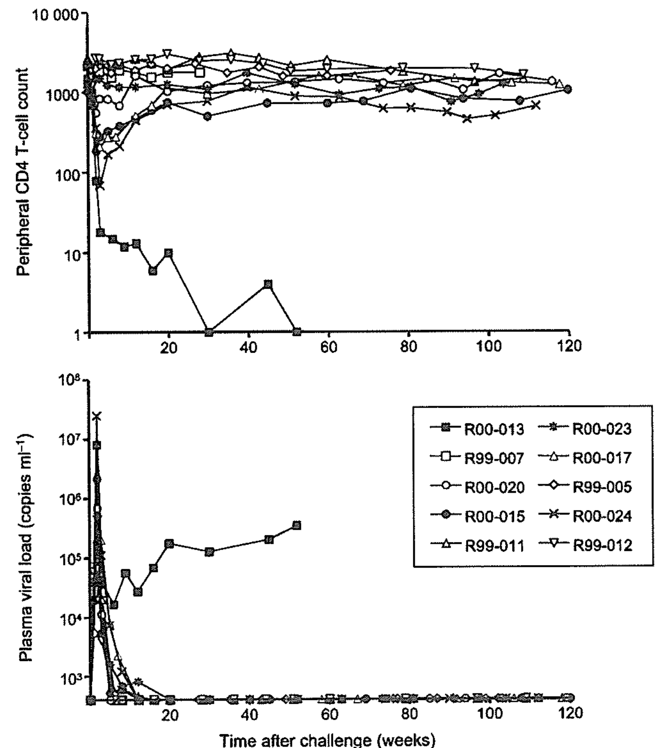

Fig. 1. Follow-up of vaccinated macaques after SHIV89.6PD challenge. Macaque R00-013 was a non-controller that failed to control virus replication, with acute $CD4^+$ T-cell depletion, whereas the other nine animals were SHIV controllers. (a) Peripheral $CD4^+$ T-cell counts μl^{-1}. (b) Plasma viral loads [viral RNA copies (ml plasma)$^{-1}$].

in amplifying almost the entire coding region of the proviral genomes from three (R00-015, R00-017 and R00-023) of the eight controllers at around 1 year for sequencing and found no dominant non-synonymous mutations except for one leading to a change in aa 401 in Env in macaque R00-015, suggesting inefficient virus replication during the period of SHIV control.

Virus-specific T-cell responses

We next examined changes in virus-specific T-cell frequencies during the period of SHIV89.6PD control. The SHIV controllers did not rapidly lose SHIV-specific T cells but most showed a gradual decrease in SHIV-specific T-cell levels, except for macaque R99-011, which maintained constant SHIV-specific $CD8^+$ T-cell levels (Fig. 2). Thus, none of the SHIV controllers showed a significant increase in SHIV-specific T-cell levels, suggesting stable virus control without any sign of a virus replication burst in the chronic phase.

In addition to virus-specific T-cell levels, we examined epitopes that were recognized by CTLs. We focused on two SHIV controllers, R00-015 and R00-017, that were vaccinated with SeV-Gag alone and examined CTL responses

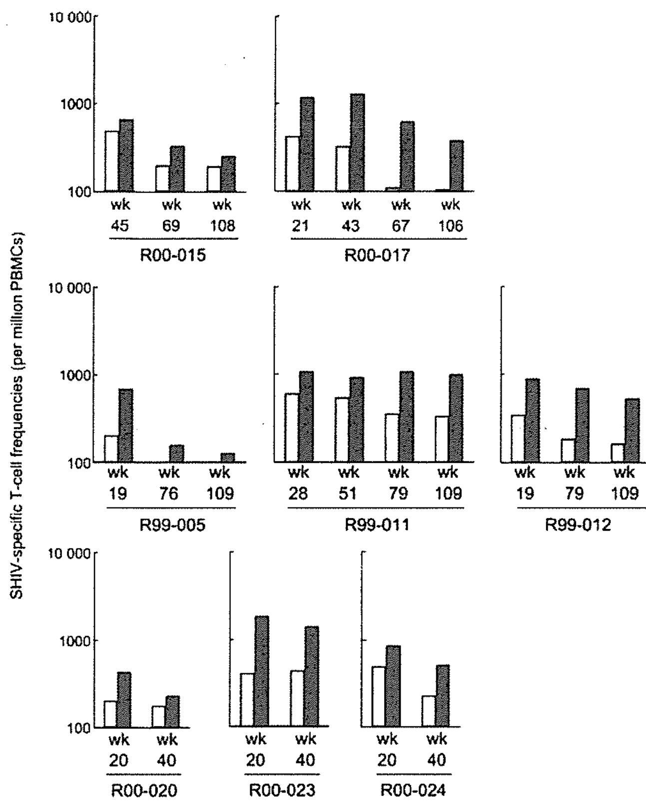

Fig. 2. SHIV-specific T-cell levels in SHIV controllers at various time points after challenge. The frequencies of SHIV-specific $CD4^+$ T cells (open bars) and $CD8^+$ T cells (shaded bars) in PBMCs are shown.

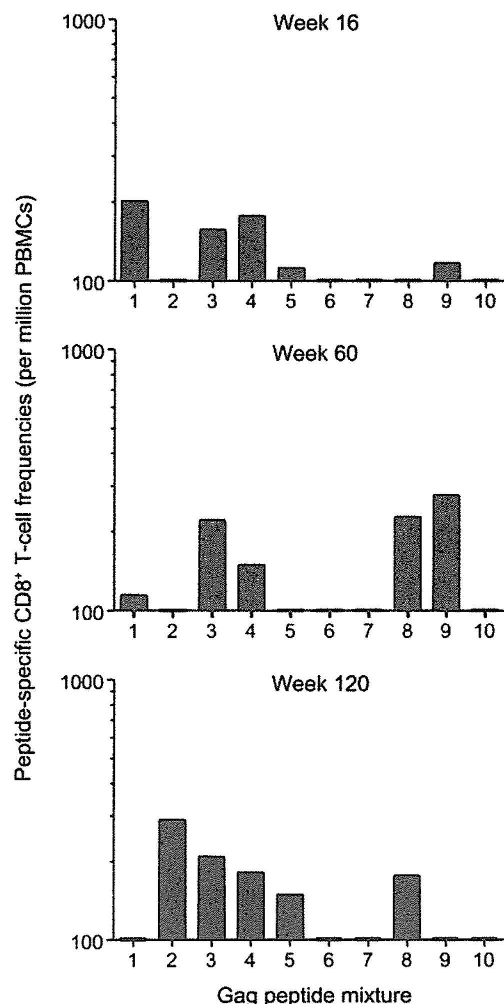

Fig. 3. Changes in frequencies of $CD8^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-015 during the period of virus control. The frequencies at week 16 (top panel), week 60 (middle panel) and week 120 (bottom panel) after SHIV89.6PD challenge are shown. A panel of overlapping peptides spanning the entire SIV Gag sequence was divided into ten pools: 1 (aa 1–65), 2 (aa 55–114), 3 (aa 104–165), 4 (aa 155–213), 5 (aa 202–265), 6 (aa 255–316), 7 (aa 306–364), 8 (aa 354–416), 9 (aa 406–464) and 10 (aa 453–510). Each pool was used for stimulation to detect peptide-pool-specific $CD8^+$ T cells.

specific for ten pools of Gag-overlapping peptides. In macaque R00-015, significant Gag peptide pool 1-specific $CD8^+$ T-cell responses were detected at week 16 but became undetectable by week 120, whereas pool 8- and 2-specific $CD8^+$ T-cell responses that were undetectable at week 16 appeared at week 60 or 120, respectively, and pool 3- and 4-specific $CD8^+$ T-cell responses were detectable throughout the observation period (Fig. 3). A similar pattern of disappearance (pool 10-specific), appearance (pool 3- and 9-specific) and maintenance (pool 6- and 8-specific) of $CD8^+$ T-cell responses during the period of SHIV control was also observed in macaque R00-017 (Fig. 4). These results suggested that SHIV89.6PD replication was not completely contained in these macaques.

Virus-specific neutralizing antibody responses

We next examined virus-specific neutralizing antibody responses by determining the end-point plasma titres required to neutralize the replication of 10 $TCID_{50}$ of virus on MT4 cells. Our vaccine regimens did not utilize Env as an immunogen and no neutralizing antibody responses were induced before challenge in any of the vaccinees, as expected. Remarkably, however, SHIV89.6PD-specific neutralizing antibodies appeared rapidly between weeks 3 and 6 post-challenge and were maintained during the observation period in all of the SHIV controllers (Fig. 5). In contrast to such efficient induction of neutralizing antibodies in SHIV controllers, macaque R00-013, which failed to control SHIV replication, showed no neutralizing antibody induction after challenge.

DISCUSSION

Long-term control of X4-tropic pathogenic SHIV has been reported in follow-up studies of several pre-clinical AIDS

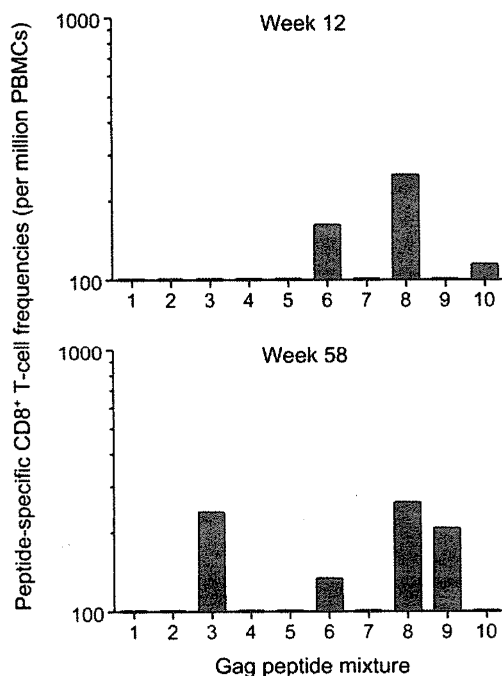

Fig. 4. Changes in frequency of $CD8^+$ T cells specific for pools of Gag peptides in PBMCs of macaque R00-017 during the period of virus control. The frequencies at week 12 (top panel) and week 58 (bottom panel) after SHIV89.6PD challenge are shown. Ten pools of Gag peptides were used for stimulation to detect peptide pool-specific $CD8^+$ T cells, as described in the legend to Fig. 3.

vaccine trials (Willey *et al.*, 2003; Sadagopal *et al.*, 2005). Whilst these vaccine regimens utilized Env as an immunogen (Amara *et al.*, 2002), we have developed vaccine regimens not targeting Env and demonstrated their efficacies leading to control of SHIV89.6PD replication in rhesus macaques (Matano *et al.*, 2001; Takeda *et al.*, 2003). In the present study, we followed up these SHIV controllers for more than 2 years after challenge. All maintained this control with undetectable plasma viraemia, indicating that efficient CTL induction by a prophylactic AIDS vaccine not targeting Env can result in sustained control of virus replication and protection from AIDS progression in a model of X4-tropic SHIV infection.

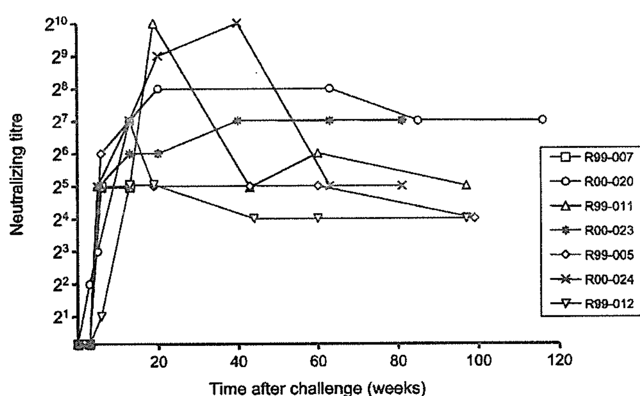

Fig. 5. SHIV89.6PD-specific neutralizing antibody levels in plasma of SHIV controllers. Plasma titres that neutralized replication of 10 $TCID_{50}$ SHIV89.6PD in seven out of nine SHIV controllers are shown. In the remaining SHIV controllers, R00-015 and R00-017, we confirmed induction of neutralizing antibodies at weeks 5, 12 and 20, but their titres were not determined.

X4-tropic SHIV and R5-tropic SIV target different $CD4^+$ T-cell subsets in rhesus macaques and this difference has been indicated as resulting in their divergent clinical courses (Nishimura *et al.*, 2004). Indeed, it has been shown that X4-tropic SHIV targets $CXCR4^+$ naive $CD4^+$ T cells for depletion, whereas R5-tropic SIV, like HIV-1 infection in humans, eliminates $CCR5^+$ effector memory $CD4^+$ T cells in rhesus macaques during the acute phase of infection (Picker *et al.*, 2004; Li *et al.*, 2005; Mattapallil *et al.*, 2005; Nishimura *et al.*, 2005; Picker & Watkins, 2005). In the latter chronic AIDS model, several CTL vaccine trials have recently shown partial reductions in viral loads with amelioration of acute memory $CD4^+$ T-cell loss, but this partial control was transient and unstable (Letvin *et al.*, 2006; Mattapallil *et al.*, 2006; Wilson *et al.*, 2006). In our previous study (Matano *et al.*, 2004), SIV control was observed consistently in the three vaccinees possessing MHC I haplotype *90-120-Ia*, but this control was not stable and two of them lost viraemia control around week 60 after challenge. In the present study showing long-term, stable SHIV control, we found several differences between X4-tropic SHIV controllers and R5-tropic SIV controllers.

First, patterns of *de novo* neutralizing antibody induction were completely different between the two. Although the vaccine regimens did not target Env, SHIV-specific neutralizing antibodies appeared rapidly and became detectable by week 6 post-challenge in the SHIV controllers, whereas no neutralizing antibody induction was observed in the SHIV non-controllers. Thus, SHIV-specific neutralizing antibodies can be rapidly induced if animals are protected by CTLs from complete $CD4^+$ T-cell depletion in the acute phase and may be involved in viraemia control at the set point and after (Rasmussen *et al.*, 2002). In contrast, SIV-specific neutralizing antibody induction in the SIV controllers was poor and less efficient than the SIV non-controllers (data not shown), indicating that neutralizing antibody responses are not involved in SIV control.

Secondly, all of the SIV controllers showed rapid selection of viral CTL escape mutations, whereas this sign of particular CTL pressure (Borrow *et al.*, 1997; Goulder *et al.*, 1997; Price *et al.*, 1997; Goulder & Watkins, 2004; Matano *et al.*, 2004) was not observed in any of the SHIV controllers. Additionally, SIV control was associated with some MHC haplotypes such as *90-120-Ia*, but SHIV control was observed in vaccinees with diverse MHC haplotypes. Indeed, none of the SHIV controllers had the MHC

haplotype *90-120-Ia* associated with SIV control. Although the involvement of functional virus-specific CD4^{+} T-cell responses remains unclear, these results support the notion that multiple target-specific CTL effectors are involved in SHIV control, whereas relatively limited regions of viral antigens are targeted by effectors responsible for SIV control.

All of the SHIV controllers maintained virus control for more than 2 years. Sequencing of viral genomes revealed a predominance of the wild-type sequence around 1 year after SHIV89.6PD challenge, and analysis of SHIV-specific T-cell levels showed no signs of a burst of virus replication during the chronic phase. These results indicated stable virus control in the chronic phase in the SHIV controllers. Interestingly, however, analysis of Gag peptide-specific CD8^{+} T-cell responses in some of the SHIV controllers showed a shift of targeting epitopes during the period of virus control, suggesting that virus replication was inefficient but not completely contained, even in the SHIV controllers.

In summary, the present study revealed several differences in vaccine-based virus control in a model of X4-tropic SHIV compared with R5-tropic SIV infections. Our results suggest that, compared with virus control with limited effectors in SIV controllers, the control of X4-tropic SHIV89.6PD replication may be maintained more stably in the presence of multiple functional immune effectors.

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BCG vaccine elicits both T-cell mediated and humoral immune responses directed against mycobacterial lipid components

Yoshiyuki Watanabe [a,b], Eiji Watari [a], Isamu Matsunaga [c], Kenji Hiromatsu [d], Christopher C. Dascher [e], Tetsuo Kawashima [b], Yoshihiko Norose [a], Kazuo Shimizu [b], Hidemi Takahashi [a], Ikuya Yano [f], Masahiko Sugita [a,c,*]

[a] *Department of Microbiology and Immunology, Nippon Medical School, Tokyo 113-8602, Japan*
[b] *Department of Surgery, Nippon Medical School, Tokyo 113-8602, Japan*
[c] *Laboratory of Cell Regulation, Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[d] *Department of Infectious Diseases, Miyazaki Medical College, Miyazaki 889-1692, Japan*
[e] *Department of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston MA02115, USA*
[f] *BCG Laboratory, Tokyo 204-0022, Japan*

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Abstract

The universe of antigens recognized by αβ T cells has recently been expanded to include not only major histocompatibility complex (MHC)-presented protein antigens but also CD1-presented lipid antigens. The significance of lipid-reactive T cells in host defense has been appreciated, using the guinea pig model of human tuberculosis. Here, we show that immunization with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), the commonly used anti-tuberculosis vaccine, induces activation of guinea pig cytotoxic T cells recognizing BCG lipids in the context of CD1 molecules. Further, BCG-immunized, but not mock-immunized, guinea pigs mount IgG antibody responses directed against lipoarabinomannnan, an essential cell wall lipid component of mycobacteria. These observations emphasize the ability of BCG to activate the host adaptive immunity to mycobacteria-derived lipids, which could potentially contribute to protection against tuberculosis.
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Keywords: BCG; Lipid; CD1; Guinea pigs

1. Introduction

Despite recent advances in the development of specific antimicrobial agents, *Mycobacterium tuberculosis*, a causative microorganism of tuberculosis, is still a tremendous threat to mankind. It is estimated that about one third of the world's population is currently infected with this microorganism and tuberculosis kills more than 2 million people each year [1]. While extensive effort has been made to develop new vaccines that may confer effective immune protection from tuberculosis, bacillus Calmette-Guerin (BCG) is still the only anti-tuberculosis vaccine widely available for humans at present. This live, attenuated vaccine derived from a virulent strain of *Mycobacterium bovis*, has been used over 80 years, and its protective effect has been well appreciated especially against miliary tuberculosis and tuberculous meningitis in the childhood although its ability to confer protective immunity against pulmonary tuberculosis in the adult remains controversial [2].

BCG vaccination prominently elicits activation of MHC class II-restricted, $CD4^+$ T cells that recognize mycobacteria-derived protein antigens, and apparently, a fraction of protein antigen-specific, MHC class II-restricted $CD4^+$ T cells can mediate protection against tuberculosis as evidenced by studies of MHC class II-deficient mice, in which absence of conventional $CD4^+$ T cells is accompanied by increased susceptibility to mycobacterial infection [3,4]. In addition,

* Corresponding author at: Tel.: +81 75 751 4028; fax: +81 75 752 3232.
E-mail address: msugita@virus.kyoto-u.ac.jp (M. Sugita).

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a significant contribution of MHC class I-restricted $CD8^+$ T cells in clearing mycobacterial infection has also been demonstrated in mice, but it has often proved very difficult to identify the $CD8^+$ T cell response in humans [5,6].

Besides protein antigen-specific, MHC-restricted T cells, recent studies have identified a distinct T cell population in humans, but not in mice, which recognizes mycobacteria-derived lipid antigens in the context of group 1 CD1 molecules (CD1a, b, c) [7–12]. Given that these CD1 molecules are expressed on dendritic cells (DCs) and a fraction of activated macrophages, the two major reservoir cell types for mycobacteria, group 1 CD1-dependent activation of specific T cells may occur during the course of mycobacterial infection to mediate protective immunity [13–15]. Indeed, group 1 CD1-restricted $CD8^+$ T cell lines were isolated from healthy subjects as well as patients with mycobacterial infection, and their outstanding ability to detect and lyse mycobacteria-infected $CD1^+$ cells has been noted [16,17]. These observations emphasize the possibility that the human adaptive immunity against mycobacterial infection may involve immune recognition of both protein and lipid antigens derived from mycobacteria.

As a live vaccine, BCG produces not only protein antigens but also lipid antigens, but the ability of the BCG vaccine to induce the lipid-specific immune response remains to be assessed. Thus, we previously set up a study of BCG-immunized human subjects and found that these individuals contained a significant circulating pool of $CD8^+$ T cells that recognized BCG-infected DCs in a CD1-dependent, but MHC-independent, fashion [18]. However, because of limitations in human studies, it was difficult to separate T cell responses induced specifically by BCG vaccination from those potentially resulting from natural infection with other mycobacteria species. To precisely monitor the lipid antigen-specific immune response induced by BCG, we chose guinea pigs as model animals, which, unlike mice and rats, have evolved a set of CD1 proteins that are comparable in function to human group 1 CD1 molecules [19–21]. Using these animals, we show in the present study that the BCG vaccine specifically induces both T-cell mediated and humoral immune responses directed against mycobacterial lipids.

2. Materials and methods

2.1. Animals

Four- to five-week-old female inbred strain 2 guinea pigs were purchased from Japan SLC, Inc. (Shizuoka, Japan), and bred in the animal facilities either at Nippon Medical School or at the Institute for Virus Research of Kyoto University. Animals were housed under specific pathogen-free conditions and all animal experiments were performed according to the Guidelines on Animal Welfare approved by the committee on animal use at the corresponding institutions.

2.2. BCG culture and inoculation

The Tokyo 172 strain of BCG, grown at 37 °C in 7H9 medium, was harvested at its midlog phase growth, washed and suspended in phosphate-buffered saline (PBS). Without prior sonication, the suspension was passed through a 5-μm pore size filter to obtain single-cell bacteria. The viability of bacteria was constantly >90%. For immunization of guinea pigs, 2×10^7 colony forming units were injected intradermally in each upper hind leg.

2.3. Antigens

The total lipid fraction of BCG was obtained by extraction with chloroform/methanol as described previously [22]. Methyl ketomycolate (keto-MA), methyl methoxymycolate (methoxy-MA), methyl α-mycolate (alpha-MA), sulfolipid (SL), trehalose 6,6′-dimycolate (TDM) and lipoarabinomannan (LAM) derived from *M. tuberculosis* (Aoyama-B) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Mycobacterial culture filtrate proteins (CFP) and *Mycobacterium phlei*-derived glucose monomycolate (GMM) were kindly provided from Drs. John T. Belisle (Colorado State University) and D. Branch Moody (Harvard Medical School), respectively.

2.4. Antibodies

Mouse monoclonal antibodies to guinea pig CD8 (CT6) and CD4 (CT7) were purchased from Serotec (Raleigh, NC). A rat anti-guinea pig CD4 antibody (H155) [23], a rabbit polyclonal antibody specific for LAM [24], a mouse anti-guinea pig CD1 antibody (CD1F2/6B5) [21] as well as a mouse negative control antibody (RPC5.4) [18] have been described elsewhere.

2.5. Isolation of T cells and bone marrow-derived dendritic cells

T cells were enriched from the spleens of immunized or unimmunized guinea pigs by passage through nylon wool fiber columns, and were labeled with either rat anti-guinea pig CD4 antibody (H155) or mouse anti-guinea pig CD8 antibody (CT6). The labeled cells were then removed by two cycles of separation with Dynabeads (Dynal Biotech, Lake Success, NY) coupled with goat antibodies to IgG of the corresponding species to obtain $CD4^+$ T cell-depleted and $CD8^+$ T cell-depleted populations, respectively. Flow cytometric analysis revealed that the percentage of $CD8^+$ T cells contained in the $CD8^+$ T cell-depleted population and the percentage of the $CD4^+$ T cells contained in the $CD4^+$ T cell-depleted population were constantly less than 1.2%.

Bone marrow-derived dendritic cells (BM-DCs) were isolated as described previously [20]. Briefly, guinea pig femurs and tibias were isolated aseptically and flushed by syringe with RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO).

The mononuclear cells in the suspension were obtained by centrifugation at $400 \times g$ on a layer of density gradient medium formulated to a density of 1.107, made by combining 2.5 volumes of Histopaque 1119 (Sigma–Aldrich) and 1 volume of Ficoll Paque Plus (Amersham Biosciences, Piscataway, NJ). The isolated mononuclear cells were cultured in 150-cm^2 flasks in the presence of human granulocyte-macrophage colony stimulating factor (GM-CSF) (40 ng/ml; PeproTech, Rocky Hill, NJ) for 1 day, and non-adherent cells were then transferred to a new flask. The cultures were fed every 2 days by removing half of the medium and replacing it with fresh medium containing GM-CSF. After 10 days, non-adherent cells and loosely adherent cells, representing a population highly enriched for BM-DCs, were collected.

2.6. T cell proliferation assays

Freshly isolated T cells (1×10^5/well) were cultured with irradiated (3000 rad) BM-DCs (2×10^4/well) in triplicate in 96-well U-bottom microtiter plates in the presence or absence of the following antigens: CFP (10 μg/ml), total lipid fraction of BCG (50 μg/ml), keto-MA (2.5 μg/ml), methoxy-MA (2.5 μg/ml), alpha-MA (2.5 μg/ml), SL (2.5 μg/ml), TDM (2.5 μg/ml), GMM (2.5 μg/ml) and LAM (5 μg/ml). Cultures were incubated for 4 days at 37 °C in a 5% CO_2 incubator, pulsed with [^{3}H]thymidine (1 μCi/well; MP Biomedicals, Inc., Irvine, CA) and incubated for an additional 6 h. The plates were harvested on a Tomtec 96-well plate harvester (Wallac, Turku, Finland) and thymidine incorporation was measured with a Betaplate liquid scintillation counter (Wallac).

2.7. Flow cytometry

Cells were incubated with primary antibodies for 30 min on ice, washed twice with FACS buffer (2% heat inactivated fetal calf serum, 0.02% sodium azide in PBS), and then labeled with fluorescein isothiocyanate (FITC)-conjugated goat F(ab′)$_2$ anti-mouse IgG/IgM (BioSource International, Camarillo, CA). Subsequently, the labeled cells were analyzed, using a fluorescence-activated cell sorter (FACSVantage; BD Biosciences, San Jose, CA). Dead cells were excluded by propidium iodide.

2.8. Cytolytic assays

A CD1-negative guinea pig fibroblast cell line (104C1) stably transfected with expression vector constructs encoding either the guinea pig CD1b1, CD1b2, CD1b3 or CD1b4 proteins [25] was labeled with ^{51}Cr for 2 h, and then incubated overnight either with or without the total lipid fraction of BCG (25 μg/ml). The cells were washed extensively and used as target cells in cytolytic assays. For the source of effector T cells for primary cytotoxic T cell responses, splenic T cells from BCG-vaccinated guinea pigs were enriched by nylon wool columns and then cultured for 6 days in the presence of the total lipid fraction of BCG (25 μg/ml) and irradiated (5000 rad) nylon wool-adherent splenic cells. The cytolytic assays were performed as described [25]. The effector:target ratio used in each experiment ranged from 5:1 to 10:1, depending on the yield of the effector T cells.

2.9. Enzyme-linked immunosorbent assays (ELISA) for anti-LAM antibody

Flat-bottom Immulon two microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with purified LAM (50 ng/well) and incubated overnight at 4 °C with PBS containing 1% bovine serum albumin (Sigma–Aldrich). The LAM-coated microtiter plates were then incubated with serum samples for 1 h at room temperature, followed by incubation with horse radish peroxidase (HRP)-conjugated F(ab′)$_2$ fragment goat anti-guinea pig IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After extensive washing with PBS containing 0.05% Tween-20 (Sigma–Aldrich), color development was performed with the ImmunoPure TMB substrate kit (Pierce, Rockford, IL) according to the manufacturer's instruction, and the absorbance at 450 nm was measured. Rabbit anti-LAM antiserum and HRP-conjugated F(ab′)$_2$ fragment goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used as a positive control. The titer of anti-LAM antibody in each guinea pig sample was expressed either directly as arbitrary units based on the standard curve for the rabbit anti-LAM antiserum.

3. Results

3.1. Protein- and lipid-specific T cell proliferative responses induced by BCG

Inbred strain 2 guinea pigs were either mock treated or immunized with BCG by intradermal injection in each upper hind leg. After 2 weeks, splenic T cells were isolated, and separated into CD8$^+$ T cell depleted and CD4$^+$ T cell depleted populations. Each cell population was cultured with mycobacteria-derived antigens in the presence of MHC class II$^+$ CD1$^+$ BM-DCs, and antigen-specific responses were assessed by measuring cell proliferation.

The only statistically significant proliferation by the CD8 depleted T cell population was observed when T cells isolated from BCG-vaccinated, but not mock-treated, guinea pigs were cultured with mycobacterial culture filtrate proteins (CFP), indicating that typical MHC class II-dependent, protein antigen-specific T cell responses have been induced by the BCG vaccination protocol used for these studies (Fig. 1A).

In contrast, no significant proliferation by the CD4 depleted T cell population derived from BCG-vaccinated guinea pigs was observed in response to CFP as well as

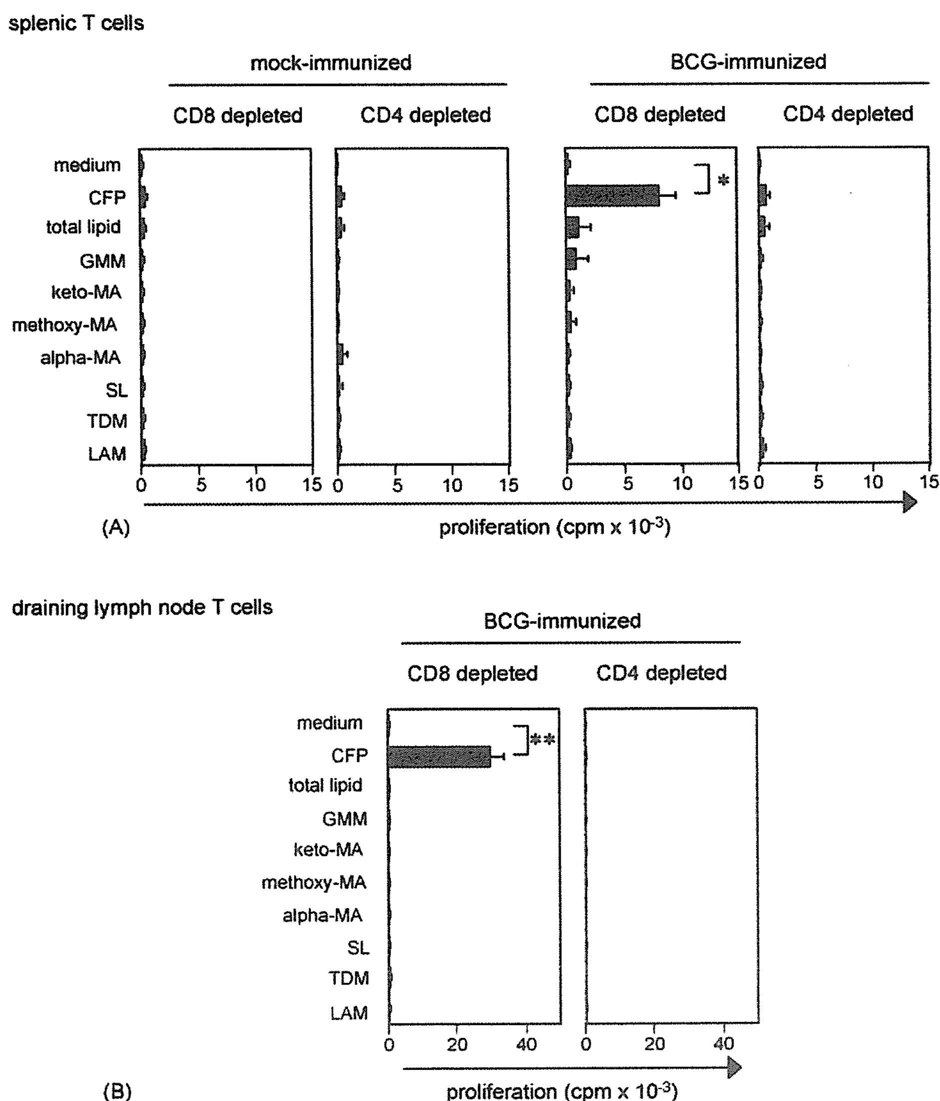

Fig. 1. T-cell proliferation in response to mycobacterial protein and lipid antigens. (A) Guinea pigs were either BCG- or mock-vaccinated ($n = 6$ for each group), and after 2 weeks, the CD8 depleted and the CD4 depleted T cell populations were obtained from the spleen. The cells were then cultured with MHC class II^+ $CD1^+$ BM-DCs in the presence or absence of the mycobacterial culture filtrate proteins (CFP), the total lipid fraction of BCG, glucose monomycolate (GMM), methyl ketomycolate (keto-MA), methyl methoxymycolate (methoxy-MA), methyl α-mycolate (alpha-MA), sulfolipid (SL), trehalose 6,6′-dimycolate (TDM) or lipoarabinomannan (LAM), and their ability to respond by proliferation to each antigen was assessed by [^{3}H]thymidine incorporation. The only significant proliferation was observed when the CD8 depleted T cell population was cultured in the presence of CFP ($^*p < 0.01$). Experiments were carried out three times to confirm the results. (B) Draining lymph node T cells were also obtained from three guinea pigs of each group described above, and separated into the CD8 depleted and CD4 depleted T cells. The cells were then cultured with BM-DCs in the presence or absence of each of the indicated antigens, and their proliferative responses were assessed. The only significant proliferation was observed when the CD8 depleted T cell population was cultured in the presence of CFP ($^{**}p < 0.01$). Experiments were carried out twice to confirm the results.

the total lipid fraction of BCG (Fig. 1A). Since the total lipid fraction contained a mixture of low concentrations of specific lipids that might not be sufficient for T cell activation, an array of purified mycobacterial lipid and glycolipid components, including glucose monomycolate and lipoarabinomannan that were known to bind human CD1, was also tested. Even at concentrations high enough for human T cell stimulation, however, splenic T cells (Fig. 1A) and draining lymph node T cells (Fig. 1B) from BCG-vaccinated guinea pigs did not proliferate significantly in response to any of these purified antigens.

3.2. *Lipid-specific cytotoxic T cells activated by the BCG vaccine*

Since it sometimes proves difficult to identify $CD8^+$ T cell responses in cell proliferation assays, we then switched to cytolytic assays. As target cells in these assays, we prepared a strain 2 guinea pig fibroblast cell line (104C1) stably transfected with expression vector constructs encoding the guinea pig CD1b1, CD1b2, CD1b3 or CD1b4 isoforms. Flow cytometric analysis with an antibody (CD1F2/6B5) recognizing all the guinea pig CD1 isoforms showed

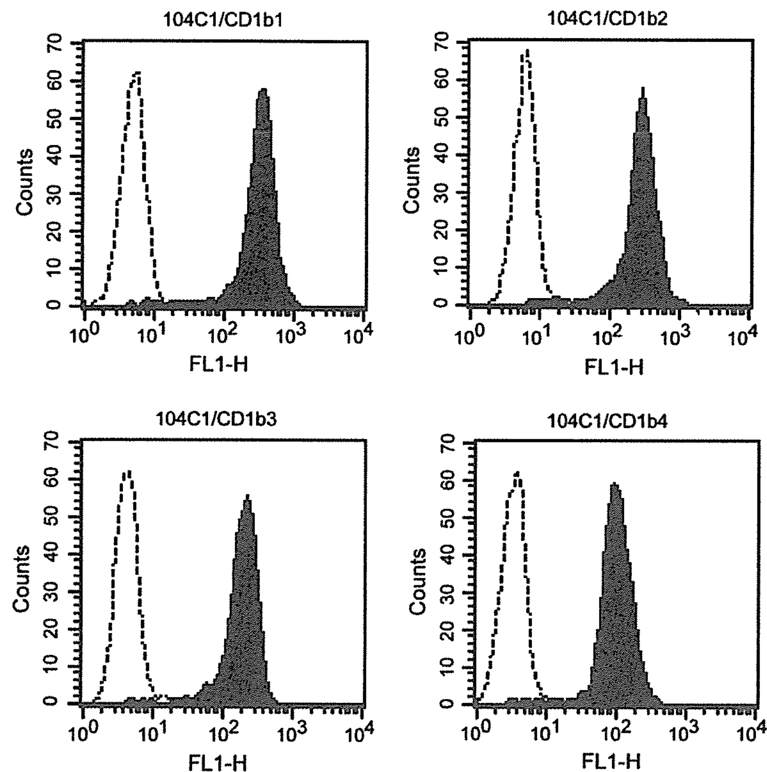

Fig. 2. Flow cytometric analysis of a guinea pig fibroblast cell line (104C1) stably transfected with either CD1b1, CD1b2, CD1b3 or CD1b4 cDNAs. The surface expression of each guinea pig CD1 isoform was detected with the anti-pan CD1 antibody (CD1F2/6B5) that recognized all the known guinea pig CD1 isoforms.

that each transfectant cell line expressed comparable levels of the transfected CD1 isoform on the cell surface (Fig. 2).

Splenic T cells derived from BCG-vaccinated guinea pigs specifically killed 104C1 transfectant cells expressing either CD1b2, CD1b3 or CD1b4 only when these target cells were pulsed with the total lipid fraction of BCG (Fig. 3). In contrast, no specific killing activity was detected against antigen-pulsed 104C1 cells expressing CD1b1. These results indicated that BCG vaccination elicited cytotoxic T cell responses directed against BCG-derived lipid components and that CD1b2, CD1b3 and CD1b4 functioned as restriction elements for these T cell responses. Interestingly, our previous studies detected distinct intracellular distribution patterns between guinea pig CD1b3 and CD1b4 proteins that were similar to those for human CD1a and CD1b isoforms [21,26]. The CD1b3- and CD1b4-restricted T cell responses to BCG lipids in guinea pigs may represent the human CD1a- and CD1b-restricted cytotoxic T cell responses that we detected previously in the circulating $CD8^+$ T cell pool of BCG-vaccinated people [18].

3.3. Lipid-specific humoral immunity induced by BCG

It has been recently suggested that, besides T-cell mediated immune responses, humoral immune responses to mycobacterial lipid components may be elicited upon infection with mycobacteria, which could potentially contribute to the host defense [27,28]. Therefore, we examined the possibility that production of antibodies of the IgG class that were specific for mycobacterial lipid antigens might be induced following BCG vaccination.

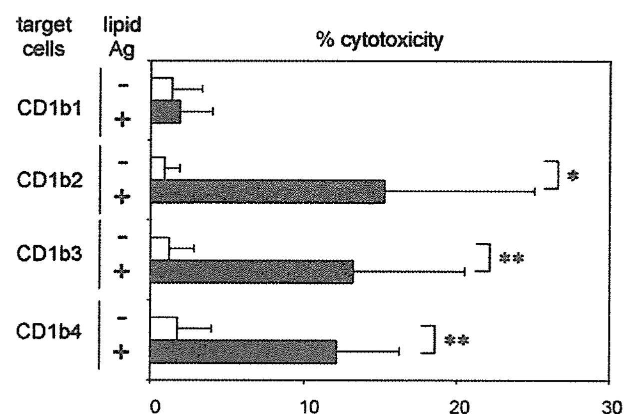

Fig. 3. CD1-dependent recognition of lipid antigen-pulsed target cells by cytotoxic T cells. Six guinea pigs were vaccinated with BCG, and splenocytes obtained after 2 weeks were tested for their ability to lyse 104C1 transfectants expressing either CD1b1, CD1b2, CD1b3 or CD1b4 isoforms in ^{51}Cr release assays. CD1b2-, CD1b3- and CD1b4-expressing cells were specifically killed by T cells only when the target cells were pulsed with BCG-derived lipid antigens ($^{*}p<0.05$; $^{**}p<0.01$). Experiments were carried out three times to confirm the results.

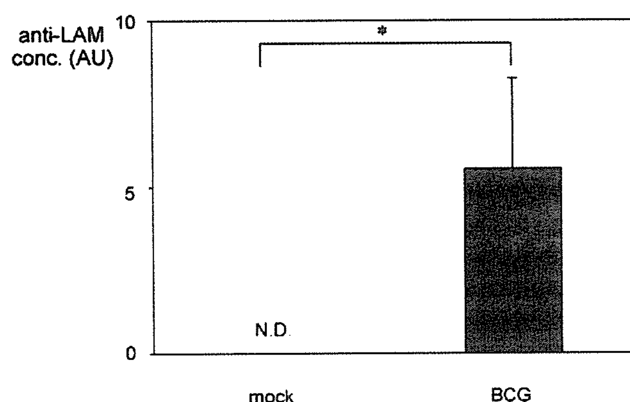

Fig. 4. Induction of IgG anti-LAM antibody production by BCG. Guinea pigs were either BCG- or mock-vaccinated ($n = 5$ for each group), and after 4 weeks, serum samples were collected and tested for IgG anti-LAM antibodies by ELISA. The titers were expressed in arbitrary units (AU) calculated based on the standard curve for the reference anti-LAM antiserum. Note that, whereas no specific IgG antibodies to LAM were detected (N.D.) in sera from any of mock-immunized guinea pigs, BCG vaccinated guinea pigs mounted the IgG antibody response to LAM significantly ($^{*}p < 0.01$). Experiments were carried out three times to confirm the results.

Guinea pigs were either BCG- or mock-vaccinated, and after 4 weeks, the sera were collected and tested for IgG antibodies against LAM. As shown in Fig. 4, whereas none of the mock-vaccinated guinea pigs mounted detectable levels of IgG responses to LAM, a significant production of the IgG anti-LAM antibodies was detected in all the BCG-vaccinated guinea pigs. These results indicated that BCG vaccination was capable of inducing mycobacterial lipid-specific IgG responses.

4. Discussion

As facultative intracellular pathogens, mycobacteria survive and replicate in the phagosome of macrophages and DCs by inhibiting phagosome acidification and phagosome-lysosome fusion [29,30]. Protein antigens produced in phagosome-resident mycobacteria are unlikely to gain easy access to the cytosol for MHC class I presentation or the lysosome for MHC class II presentation [31]. In contrast, lipid antigens are inserted into the phagosomal membrane and traffic to endocytic compartments which group 1 CD1 molecules sample, enabling efficient monitoring of mycobacteria-infected cells by the CD1 system [26,32–34]. Previously, most studies have examined the group 1 CD1-mediated T cell responses to *M. tuberculosis*. Here, we provide further evidence that responses to lipid antigens are also induced after BCG vaccination in a small animal model that expresses group 1 CD1 molecules. Since these lipid-specific T cell responses are known to be a critical component of the host defense against tuberculosis [35], the present study suggests that the vaccine effect of BCG may be accounted for at least in part by its ability to induce T cell responses directed against mycobacterial lipid antigens.

The cellular pathways for BCG-induced activation of CD1-restricted T cells remain to be determined. Initial studies focusing on $CD1^{+}$ DCs demonstrated that group 1 CD1-restricted T cell lines were efficiently activated by *M. tuberculosis*-infected DCs [17,36]. This pathway directly triggered by infected DCs may occur following BCG vaccination since a circulating pool of such T cells can be detected in BCG-immunized people [18]. In contrast, macrophages were previously thought not to contribute to activation of lipid-specific T cells because of the apparent lack of group 1 CD1 expression [37]. Recent evidence has suggested, however, that a sizable fraction of BCG-infected macrophages gains the ability to express group 1 CD1 molecules and to mediate stimulation of CD1-restricted T cells [38,39]. Given that macrophages are the major host cell type for mycobacterial infection, this pathway may play a significant role in BCG-induced T cell responses to lipids. Alternatively, some BCG-infected macrophages are prone to undergo apoptosis, and the apoptotic vesicles containing BCG are taken up by $CD1^{+}$ DCs, resulting in activation of lipid-specific T cells [40,41]. These three distinct pathways may or may not occur simultaneously, depending on the dose of BCG and which cell types are available at the site of immunization.

Besides T-cell mediated immune responses to lipids, the present study indicates that antibody responses to lipids are also elicited by BCG immunization. Previously, antibody responses were not considered effective for protection against intracellular pathogens, such as mycobacteria, but a protective antibody response to LAM has recently been suggested [27,42]. LAM has several immunosuppressive effects that may favor survival of mycobacteria in the host, including downregulation of DC function by interaction with the DC-SIGN receptor. Thus, it may be reasonable to speculate that neutralizing antibodies to LAM could contribute to host defense against mycobacterial infection [43–45].

A fraction of B cells in humans and virtually all B cells in guinea pigs express group 1 CD1 molecules, and double negative CD1-restricted T cells are known to exert helper activity to support IgG class antibody production [21,46]. Further, mycobacteria-derived glycolipid antigens, such as LAM, contain both T-cell and B-cell epitopes [12], suggesting an interesting possibility that production of IgG antibodies to some lipid antigens may be dependent on group 1 CD1 molecules. We speculate that LAM released to the extracellular space from BCG-infected cells may be captured by $CD1^{+}$ B cells expressing specific surface immunoglobulins and activate LAM-reactive, CD1-restricted T cells, which support class switching to IgG in B cells via cognate interactions.

The successful induction of protein antigen-specific, MHC class II-restricted T cell responses by BCG has been conveniently monitored by the tuberculin test detecting the delayed type hypersensitivity response to purified protein derivatives (PPD). It is considered unlikely, however, that the positive PPD test directly correlates with protective immunity against tuberculosis [47]. The lipid-specific immune responses detected in the present study may be critical for

a complete understanding of BCG-induced protective immunity against tuberculosis.

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Identification of a Novel Circulating Recombinant Form (CRF33_01B) Disseminating Widely Among Various Risk Populations in Kuala Lumpur, Malaysia

Kok Keng Tee, MMedSc,† Xiao-Jie Li, MD, PhD,* Kyoko Nohtomi, BSc,* Kee Peng Ng, MBBS, PhD,‡ Adeeba Kamarulzaman, MBBS, FRACP,† and Yutaka Takebe, MD, PhD**

Summary: A molecular epidemiological investigation was conducted among various risk populations (n = 184) in Kuala Lumpur, Malaysia, in 2003 to 2005, on the basis of nucleotide sequences of protease and reverse transcriptase regions. In addition to circulating HIV-1 strains, including CRF01_AE (57.1%), subtype B (20.1%), and subtype C (0.5%), we detected a candidate with a new circulating recombinant form (CRF). We determined four near-full-length nucleotide sequences with identical subtype structure from epidemiologically unlinked individuals of different risk and ethnic groups. In this chimera, two short subtype B segments were inserted into the *gag*-RT region in a backbone of CRF01_AE. The recombinant structure was distinct from previously identified CRF15_01B in Thailand. In agreement with the current HIV nomenclature system, this constitutes a novel CRF (CRF33_01B). The overall prevalence of CRF33_01B is 19.0% (35/184). Although the prevalence of CRF33_01B is particularly high among injecting drug users (42.0%, 21/50), it is also detected in a substantial proportion of homo-/bisexual males (18.8%, 3/16) and heterosexuals (9.8%, 9/92). Moreover, unique recombinant forms composed of CRF01_AE and subtype B that have a significant structural relationship with CRF33_01B were detected in 1.6% (3/184) of study subjects, suggesting an ongoing recombination process in Malaysia. This new CRF seems to be bridging viral transmission between different risk populations in this country.

Key Words: HIV-1 circulating recombinant form (CRF), unique recombinant form (URF), CRF01_AE, subtype B, CRF33_01B, Malaysia, Asia

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From the *Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan; †Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; and ‡Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

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Reprints: Yutaka Takebe, MD, PhD, Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan (e-mail: takebe@nih.go.jp).

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Since the first case of AIDS was reported in Malaysia in 1986,[1] a total of 67,438 HIV infections had been identified nationwide by the Ministry of Health by June 2005. Although the adult HIV prevalence rate remains low (0.5%:0.2%–1.5%), elements exist that could cause the epidemic to erupt suddenly. In 2004 alone, an average of 18 new cases of HIV infections was reported daily. The Joint United Nations Program on HIV/AIDS (UNAIDS) estimated that approximately 69,000 people in Malaysia were living with HIV in 2005.[2] The main route of HIV transmission in Malaysia is through injecting drug use, which accounts for 75% of all reported HIV cases (Fig. 1, inset). The growing proportion of HIV cases attributed to sexual transmission (17% in 2002 compared with 11.5% in 2001 and 7% in 1995; 46% increase between 2001 and 2002), suggesting that HIV is spreading in the general population.[3,4]

HIV-1 exhibits a tremendous genetic diversity that is driven by high rates of mutation (3×30^{-5} sites/genome/replication cycle) and recombination (2–3 crossovers/replication cycle), coupled with high viral turnovers (>10^9 per day) and persistent nature of infections (300 replication cycles/year).[5] By these mechanisms, HIV-1 group M, which is largely responsible for the global pandemic, has diversified into 11 subtypes and sub-subtypes (A1, A2, B, C, D, F1, F2, G, H, J, and K) and various types of recombinants (http://hiv-web.lanl.gov/). HIV-1 recombinants with epidemic spread are known as circulating recombinant forms (CRFs). Thirty-two CRFs are currently recognized.[6] Four CRFs have been reported so far in Asia: CRF01_AE and CRF15_01B in Thailand and CRF07_BC and CRF08_BC in China. To define a CRF, at least three epidemiologically unlinked HIV-1 sequences with identical mosaic structures should be characterized, at least two of them in near-full-length genomes (>8 kb).[7] In addition to CRFs, various types of unique recombinant forms (URFs) that were detected in a single individual or a single epidemiologically linked cluster have been identified in the region, where the multiple lineages of HIV-1 strains cocirculate in the same population.

In the early phase of the Thai epidemic, two HIV-1 strains—CRF01_AE and subtype B′ (Thai variant of subtype B)—were circulating relatively independently among different risk populations. CRF01_AE was distributed among persons at risk of sexual exposure, while subtype B′ was distributed mainly among injecting drug users (IDUs).[8,9] However, by 1999 it was reported that CRF01_AE accounted for >50% of new infections among IDUs.[10–12] Cocirculation of CRF01_AE

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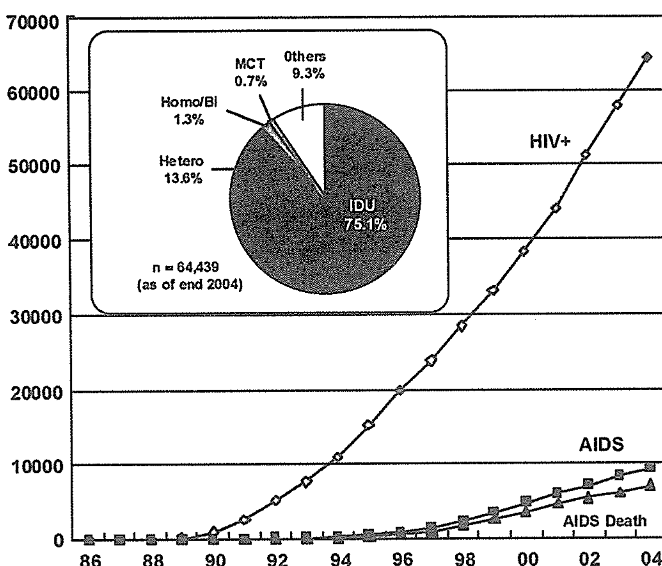

FIGURE 1. Status and distribution of HIV-1 infection in different risk categories in Malaysia. Cumulative numbers of HIV-1 infections, AIDS cases, and AIDS-related deaths reported by the Ministry of Health in Malaysia (1986–December 2004). Inset, Distribution of HIV-1 infections in different risk categories as of the end of 2004 (n = 64,439). Abbreviations: IDU, injecting drug users; Hetero, heterosexuals; Homo/Bi, male homo-/bisexual; MCT, mother-to-child transmission.

and subtype B′ in Thailand led to the generation of various forms of CRF01_AE/B′ recombinants,[13–18] including CRF15_01B.[19] A similar molecular epidemiological trend has been observed in Malaysia. Studies conducted in 1992 to 1997 showed that CRF01_AE and subtype B′ were prevalent among 81% of heterosexuals and 55% to 92% of IDUs, respectively.[20–22] However, more recent studies based on partial protease (Pro) and reverse transcriptase (RT) sequence data suggested the emergence of new forms of CRF01_AE/B recombinants in Malaysia.[23,24]

For the present study, we characterized near-full-length nucleotide sequences of CRF01_AE/B recombinants from eight epidemiologically unlinked individuals to define a novel CRF and other recombinant forms emerging in Malaysia and discuss herein their epidemiological and biological implications.

MATERIALS AND METHODS

Study Subjects and Specimens

All subjects (n = 184) were recruited between July 2003 and August 2005 in the HIV clinic of the University Malaya Medical Center (UMMC), Kuala Lumpur. This study was approved by the UMMC Medical Ethics Committee. Information on the patients' clinical and epidemiological backgrounds was collected from the HIV patient-management database. Study subjects included 50 IDUs, 92 heterosexuals, 14 male homosexuals, 2 male bisexuals, 4 persons who were infected from their mothers (mother-to-child transmission, MCT), and 22 cases with unknown risk factors. Subjects consisted of 150 adult males with an age range of 19 to 67 years old (mean: 38.2 ± 8.9 years old) and 31 adult females with an age range of 26 to 53 years old (mean: 36.0 ± 7.9 years old). Three MCT adolescents (2 males, 1 female) ranging in age from 5 to 9 years old (mean: 7.7 ± 2.3 years old) were also included. Specimens from these 184 patients were serologically determined to be HIV-1 positive. No HIV-2 infections were detected. HIV-1 genotypes were screened on the basis of the Pro and RT regions using plasma HIV-1 RNA, as described previously.[23,24]

Viral Isolation, Near-Full-Length DNA Amplification, and Sequencing

Peripheral-blood mononuclear cells (PBMCs) from selected patients were separated on Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences AB, Uppsala, Sweden). For virus isolation, PBMCs from HIV-1–positive individuals were cocultured with phytohemagglutinin-stimulated (1 μg/ml) $CD8^+$ T-cell–depleted PBMCs (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) from HIV-negative healthy donors in RPMI 1640 containing 10% fetal calf serum and interleukin-2 (20 U/mL). Virus production was detected by a virion-associated RT assay as described previously.[25] HIV-infected PBMCs were harvested, and proviral DNA was isolated with guanidine detergent (Invitrogen, Carlsbad, CA). Near-full-length viral DNA was amplified with primers pbsA (5′-AGT GGC GCC CGA ACA GG-3′; nucleotide positions relative to HXB2: 634–650) and 9KU5B (5′-GGT CTG AGG GAT CTC TAG TTA CCA G-3′; nucleotide positions relative to HXB2: 9666–9690) by the Expand Long Template PCR System (Roche Diagnostic GmbH, Penzberg, Germany), gel-purified, and TA-cloned with the pCR-XL-TOPO vector (Invitrogen).[26,27] Positive clones were selected, and the near-full-length genome of ~9.1 kb was directly sequenced with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the primer-walking method. For plasma samples, HIV-1 RNA was reverse-transcribed and nested-PCR–amplified to produce 10 overlapping fragments; directly sequenced; and then assembled to generate the near-full-length genomes. Primers were carefully designed to obtain sufficient numbers of overlapping nucleotides to ensure that recombinants were not generated by assembling the sequence fragments derived from different HIV-1 subtypes that had been amplified from an individual (primer sets and PCR parameters are available upon request).

Phylogenetic Analysis

Nucleotide sequences were aligned manually using Se-Al, version 1.0,[28] with HIV-1 reference subtypes and CRFs from the HIV database (http://hiv-web.lanl.gov/). Phylogenetic trees were constructed by the neighbor-joining method[29] based on the Kimura two-parameter model with a transition-to-transversion ratio of 2.0.[30] The reliability of the branching orders was tested by bootstrap analysis of 100 replicates. Bootscanning and informative-site analyses were performed using SimPlot, version 3.5,[31] with a sliding window of 250 nucleotides overlapped by 50 nucleotides to define the recombinant structure. The origin of each segment was analyzed by subregion neighbor-joining tree analysis.

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