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The Journal of Immunology

Ag85B of Mycobacteria Elicits Effective CTL Responses through Activation of Robust Th1 Immunity as a Novel Adjuvant in DNA Vaccine[1]

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CD4$^+$ T cells play a crucial role in CTL generation in a DNA vaccination strategy. Several studies have demonstrated the requirement of CD4$^+$ T cells for the induction of a sufficient immune response by coadministrating DNAs. In the present study we investigated the effectiveness of Ag85B of mycobacteria, which is known to be one of the immunogenic proteins for Th1 development, as an adjuvant of a DNA vaccine. HIV gp120 DNA vaccine mixed with Ag85B DNA as an adjuvant induced HIV gp120-specific Th1 responses, as shown by delayed-type hypersensitivity, cytokine secretion, and increasing HIV-specific CTL responses. Moreover, these responses were enhanced in mice primed with *Mycobacterium bovis* bacillus Calmette-Guérin before immunization of HIV DNA vaccine mixed with Ag85B DNA. Furthermore, these immunized mice showed substantial reduction of HIV gp120-expressing recombinant vaccinia virus titers compared with the titers in other experimental mice after recombinant vaccinia virus challenge. Because most humans have been sensitized by spontaneous infection or by vaccination with mycobacteria, these findings indicate that Ag85B is a promising adjuvant for enhancing CTL responses in a DNA vaccination strategy. *The Journal of Immunology,* 2005, 175: 2541–2547.

The use of adjuvant in vaccination is thought to be useful for enhancing the immune responses to various pathogens and tumors. One of the major advantages of plasmid DNA vaccination is the induction of MHC class I-restricted CTL responses through endogenous production of an Ag similar to viral infection (1). However, plasmid DNA immunization does not fully elicit cellular immune responses against infectious pathogens in some cases. Unlike viral infection, generation of CTLs after DNA vaccination appears to be critically dependent on functions of CD4$^+$ T cells, such as secretion of Th1 cytokines, which facilitate CTL expansion and activity (2), and activation of professional APCs through CD40-CD40L interaction to increase the expression of costimulatory molecules (3–5). Accordingly, simultaneous activation of CD4$^+$ T cells, especially Th1 cells, during priming is a promising strategy for the generation of substantial CTL responses when using a noninflammatory Ag expression system. In many cases, some helper epitopes are already present in a DNA vaccine, and epitope-specific CD4$^+$ Th cell responses are induced after vaccination. However, because CD4$^+$ T cell help for CTL generation does not require a pathogen including a CTL epitope, pathogen-specific CD4$^+$ T cell responses are not necessary for eliciting pathogen-specific CTL immunity (6, 7). This prompted us to use an appropriate molecule as an adjuvant for the induction of an effective CTL response by the activation of CD4$^+$ T cells.

Mycobacterium bovis bacillus Calmette-Guérin (BCG),[3] a currently available vaccine to prevent tuberculosis, is thought to have powerful immunogenic adjuvant activity that augments cell-mediated immune responses by induction of several Th1 cytokines (8). It is also well known that CFA, which contains heat-killed mycobacteria, augments immune responses by activating Th cells. However, the specific proteins that elicit Th1 immunity of BCG are not clear. One immunogenic protein that can induce a strong Th1-type immune response in hosts sensitized by BCG is Ag85B (also known as α Ag or MPT59) (9). Ag85B belongs to the Ag85 family, which participates in cell wall mycolic acid synthesis (10). Moreover, Ag85B is one of most dominant protein Ags secreted from all mycobacterial species, shows extensive cross-reactivity between different species, and has been shown to induce substantial Th cell proliferation and vigorous Th1 cytokine production in humans and mice infected with mycobacterial species, including individuals vaccinated with BCG (9). We previously showed that inoculation of Ag85B-transfected tumor cells enhances the immunogenicity of tumor-associated Ags and elicits a strong tumor-specific CTL response (11). In the present study we evaluated the effectiveness of Ag85B from *Mycobacterium kansassi* as an adjuvant for enhancing cellular immune responses induced by DNA vaccine.

Materials and Methods

Mice

In mice, unlike humans, studies using inbred and congenic strains have demonstrated different fastness against BCG infection among each strain (12). To give resistance to BCG infection, BALB/c (BCG-susceptible strain) × C3H/HeN (BCG-resistant strain; CC3HF1, H-2$^{d/k}$) female mice were used in this study. The mice were housed at the Laboratory Animal Center of Mie University School of Medicine.

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[3] Abbreviations used in this paper: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; DC, dendritic cell; DTH, delayed-type hypersensitivity; FN, fibronectin; MMC, mitomycin C; rVV, recombinant vaccinia virus.

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Plasmid

A highly efficient mammalian expression vector, pJW4303, was used for efficient expression of HIV env gp120 of the NL432 strain (pJWNL432) (13). The Ag85B expression vector pcDNA-Ag85B has been constructed by cloning a PCR product that possesses an Ag85B of *M. kansasii* open reading frame lacking a signal sequence into *Kpn*I-*Apa*I sites of pcDNA 3.1 (11).

Peptide synthesis

The peptides used in this study were an HIV-1 env helper epitope (315–329; RIQRGPGRAFVTIGK; p18) and CTL epitope (318–327; RGPGRAFVTI; p18-I10) in association with the class II MHC molecule I-A^d and the class I MHC molecule H-$2D^d$, respectively (14).

Priming to BCG

Six- to 8-wk-old female mice were primed to BCG by i.p. inoculation of 0.01 mg (dry weight) of BCG (Japan BCG Laboratory).

Immunization

Four weeks after BCG priming, groups of mice were i.m. injected four times with 100 μg of pJWNL432 mixed with or without 100 μg of pcDNA-Ag85B, and then the site of inoculation was immediately given an electric pulse by an Electric Square Porator (T820; BTX) to express both Ags of Ag85B and HIV gp120 in the same tissue, as previously described (15). Pulses were delivered to the muscle using a pair of electrode needles. Eight electric pulses of 50 V were delivered at a rate of one pulse per second. Each electric pulse was 99 ms in duration. Resistance was monitored with a graphic pulse analyzer (Optimizer 500; BTX). To test the dose dependency of Ag85B as an adjuvant, mice primed with BCG were coadministered various doses of pcDNA-Ag85B. Insufficiency of the amount of DNA by reduction of pcDNA-Ag85B was compensated for by mock DNA pcDNA3.1, the original expression vector of pcDNA-Ag85B, to equalize the total volume of administered DNA.

Immunohistochemical analysis

Immunized leg muscles were examined immunohistochemically for in vivo expression of HIV gp120 and Ag85B. Three days after injection, the tibialis anterior muscle was removed, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin wax. Serial sections were prepared and deparaffinized and then incubated with proteinase K for 5 min at room temperature (gp120) or heated by microwave oven three times for 5 min each time (Ag85B) to reactivate the Ag. After incubation with 3% H_2O_2/methanol for 30 min to quench endogenous peroxidase activity, the sections were blocked with normal serum and incubated with anti-HIV gp120 Ab (OEM Concepts) diluted 1/100 or rabbit anti-Ag85B antiserum (16) diluted 1/250 for 30 min at room temperature. Subsequently, the sections were additionally incubated with a biotinylated secondary Ab and HRP-labeled avidin-biotin complex (ABC-peroxidase staining kit Elite; Vector Laboratories). They were then reacted with 0.5% 3.3′-diaminobenzidine tetrachloride and 0.01% H_2O_2 to visualize the bound Abs. Sections incubated with an irrelevant Ab instead of the primary Ab were used as negative controls. Sections were slightly counterstained with hematoxylin.

Delayed-type hypersensitivity (DTH) responses

DTH responses to HIV were elicited by injecting 5 μg of p18 peptide into the footpad of each immunized mouse. The degree of footpad swelling 24 h after the injection was measured using a micrometer and was expressed as the mean increment ± SE of three mice per group (11).

Determination of cytokine production

Spleen cells from immunized mice (5×10^6) were cultured with 2.5×10^6 mitomycin C (MMC)-treated autologous spleen cells labeled with p18 peptide in 24-well culture plates at a volume of 2 ml. After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h, culture supernatants were collected and analyzed for IFN-γ (BioSource International) or IL-4 (Quantikine; R&D Systems) production by an ELISA according to the manufacturer's protocol.

RT-PCR

Total RNA was isolated from leg muscles of the site of immunization using TRIzol (Invitrogen Life Technologies), then reverse transcribed to cDNAs using a SuperScript system (Invitrogen Life Technologies). The resulting cDNA was amplified using TLR sequence-specific primers for 30 cycles of PCR (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min). The following primers corresponding to each TLR were used: 5′-ATGGCAGAAGAT GTGTCCG-3′ and 5′-GTCACCATGGCCAATGTAGG-3′ for TLR2, 5′-TGGATTCTTCTGGTGTCTTCC-3′ and 5′-AGTTCTTCACTTCGCAA CGC-3′ for TLR3, 5′-CTGGCATCATCTTCATTGTCC-3′ and 5′-GCTTAGCAGCCATGTGTTCC-3′ for TLR4, 5′-CAGAACCTTCCTG GCTATTGC-3′ and 5′-AGAGGTTGACCAGACCTTGG-3′ for TLR9, and 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ and 5′-CTTCTG CATCCTGTCAGCAATGCC-3′ for β-actin.

Generation of CTL effector cells

Effector cells were derived from spleen cells as precursor CTLs. $CD8^+$ T cells were purified with anti-CD8 magnetic beads (Miltenyi Biotec) by positive selection according to the recommended protocol (MACS system). Aliquots of 1×10^6 $CD8^+$ T cells were cocultured with 5×10^6 MMC-treated autologous spleen cells labeled with p18-I10 peptide at 37°C in a 5% CO_2 atmosphere. Two days after stimulation, human rIL-2 (Shionogi) was added to all wells at a final concentration of 5 ng/ml. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

MHC-matched (A20.2j) and unmatched (FBL-3) target cells (2×10^6) were incubated at 37°C in a 5% CO_2 atmosphere with or without 10 μg/ml p18-I10 peptide for 16 h. Then the target cells were washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100. All experiments were performed more than three times, and each group consisted of three mice.

Blocking of cytolysis

Blocking of cytolysis was performed by a method previously described (13). ^{51}Cr-labeled target cells were preincubated at 37°C for 20 min with anti-H-2 K^d, D^d, or L^d mAb (Meiji Institute of Health Science), and effector cells were then added. In a separate experiment, effector cells were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) at a 1/50 dilution with complement (Sigma-Aldrich) for 20 min at 37°C, and then labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Evaluation of HIV gp120-specific $CD8^+$ T cells by ELISPOT assay

The number of gp120-specific, IFN-γ-secreting cells was determined by ELISPOT assay. Briefly, 96-well nitrocellulose plates (Millipore) were each coated with 8 μg/ml anti-mouse IFN-γ mAb R4-6A2 (BD Pharmingen) in 100 μl of PBS. After overnight incubation at 4°C, the wells were washed three times with PBS. Then 100 μl of complete medium supplemented with 10% FCS was added to each well, and the plates were incubated at 37°C for 1 h. Triplicate samples of $CD8^+$ T cells separated from the spleen were plated in 2-fold dilutions from 5×10^5 to 6.25×10^4 cells/well. The p18-I10-labeled MMC-treated P815 cells were used as APCs. APCs (1×10^5) were added to each well, and the plates were incubated for 24 h in a 37°C incubator with a 5% CO^2 atmosphere. After stimulation, plates were washed intensively with PBS containing 0.05% Tween 20 and incubated overnight at 4°C with a solution of 2 μg/ml biotinylated anti-mouse IFN-γ mAb XMG1.2 (BD Pharmingen). Afterward, plates were washed with PBS containing 0.05% Tween 20 and 100 μl of streptavidin-alkaline phosphatase (Mabtech) at a 1/1000 dilution was added to each well. Spots were visualized using alkaline phosphatase color development buffer (Bio-Rad) and counted using KS ELISPOT (Zeiss).

Study of protection from vaccinia virus expressing HIV env gp120

The protective ability in immunized mice against systemic infection of recombinant vaccinia virus (rVV) was analyzed by real-time detection PCR as described previously (16). Twelve weeks after the first immunization, mice were challenged i.p. with 5×10^7 PFU of rVV carrying the HIV IIIB gp120 gene (rVV-HIV gp120). Five days after the challenge, the ovaries were harvested and homogenized, and DNA was isolated using a Genomic DNA Isolation kit (Promega). Primers (forward, 5′-GTTCCT TCGCCAACAGGTTAA-3′; reverse, 5′-ACTCGCGATCCTCAAAATG C-3′) and a TaqMan probe (5′-FAM-TTGGAAGCGCCACGGTTACAT TCACT-3′) were selected from the core 4b gene of vaccinia virus. Amplification and detection were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After incubation at 50°C for 2 min, amplification was begun at 95°C for 10 min, followed by 45 two-step cycles of 95°C for 15 s and 60°C for 60 s.

Statistical analysis

Statistical analysis was performed using Mann-Whitney's U test and the Kruskal-Wallis test. Values are expressed as the mean ± SD. A 95% confidence limit was taken as significant ($p < 0.05$).

Results

In vivo expression of HIV gp120 and Ag85B

It has been reported that $CD4^+$ T cells play a critical role in the generation of CTLs at the site of Ag presentation (17). To induce the effect of Ag85B as an adjuvant for augmentation of cellular immune responses, two DNA vaccines, encoding HIV gp120 and Ag85B, were mixed and administered simultaneously using in vivo electroporation. Three days after immunization, transgene expression was assessed by immunohistochemical analysis in serial sections. Except for slight transient inflammation, no pathological changes were detected in muscles after DNA injection and in vivo electroporation (data not shown) (15). Both HIV gp120 (Fig. 1*A*) and Ag85B (Fig. 1*B*) were observed inside bundles of muscle cells and connective tissue among the muscle fascicles in the same area. Transgene expressions were only seen in the area between the electrode needles.

Coadministration of Ag85B DNA induces strong HIV gp120-specific Th1-type immune responses

As in cases of tuberculosis, one of the important markers of Th1-mediated acquired immunity (not synonymous with protection) is the DTH response. To confirm the ability of Ag85B to induce Th1 responses against coadministered Ag, immunized mice were injected with HIV env helper epitope p18 into footpads, and HIV gp120-specific DTH responses were assessed. As shown in Fig. 2, mice coadministered pcDNA-Ag85B showed greater footpad swelling than mice not administered pcDNA-Ag85B. The effectiveness of Ag85B for inducing Th1-type immune responses to vaccine Ag was augmented by BCG sensitization. In contrast, no significant responses were observed in nonimmunized mice and immunized mice injected with a control peptide (data not shown).

Next, to determine the effect of Ag85B on the patterns of Th1/Th2 cytokine production, we quantified the production of IFN-γ and IL-4 by ELISA in supernatants obtained from 48-h cocultures of peptide-pulsed syngeneic APCs with spleen cells of immunized mice. The production of IL-4 from spleen cells stimulated by p18 in mice immunized with both pcDNA-Ag85B and pJWNL432 was much less than that in mice immunized with pJWNL432 alone. Relatively high levels of HIV gp120-specific IFN-γ production were observed in mice coadministered pcDNA-Ag85B. Furthermore, these Th1-type immune responses were clearly observed when mice were sensitized by BCG inoculation before DNA immunization (Fig. 3). These results are in accordance with the results for DTH responses against HIV gp120 in in vivo experiments and indicate that predominant HIV gp120-specific Th1 responses were induced by coadministration of pcDNA-Ag85B.

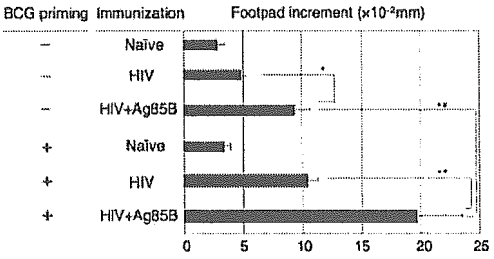

FIGURE 2. Anti-HIV gp120 DTH responses in immunized mice. BCG-primed or unprimed mice were immunized with pJWNL432 with or without pcDNA-Ag85B. The helper epitope peptide of HIV gp120 (p18) was injected into the footpads of immunized mice. The degree of footpad swelling was measured 24 h after the challenge. The results are expressed as the mean footpad increment ± SE of five mice per group. *, $p < 0.01$; **, $p < 0.001$.

Alteration of TLR mRNA expression after Ag85B DNA administration

Various proteins derived from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines (18). We therefore compared TLR mRNA expression profiles at the site of DNA injection with electroporation in pcDNA-Ag85B-immunized mice and mice immunized with pcDNA alone using RT-PCR. Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA, whereas mock immunization with electroporation resulted in only modest increases in the levels of these TLR mRNAs (Fig. 4). TLR9 mRNA was not detected in either group of mice (Fig. 4). Plasmid pcDNA3.1 has immunostimulatory sequence (six 5′-GACGTC-3′), whereas TLR9 mRNA was not detected in either group of mice under these conditions (Fig. 4). Positive reactions, however, were observed in both Ag85B-injected tissues and control tissues using high cycles (>60) of PCR (data not shown). This positive reaction was not thought to be the effect of Ag85B. These results suggested that Ag85B immunization plays a role in enhancement of the expression of these TLRs, although the possibility of indirect responses by cytokine production cannot be ruled out (18).

Ag85B enhances anti-HIV gp120-specific CTL responses

$CD8^+$ cells from BCG-primed mice and unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated in vitro with p18-I10-pulsed syngenic splenocytes, and the

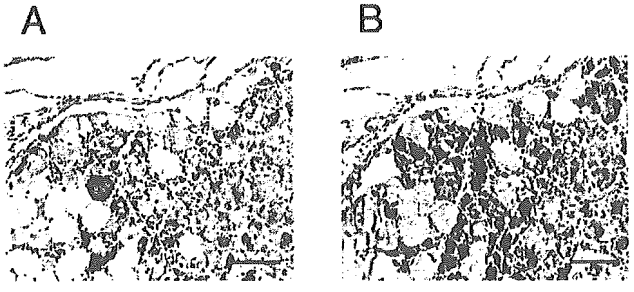

FIGURE 1. Immunostaining of serial sections of muscle tissue from a mouse 3 days after electric administration of pJWNL432 mixed with pcDNA-Ag85B. HIV gp120 (*A*) and Ag85B (*B*) were observed in muscle cells and connective tissue among the muscle fascicles in the same area. Bars represent 100 μm.

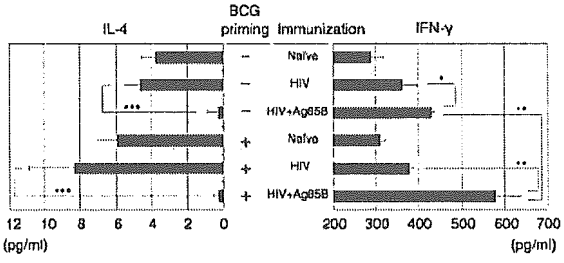

FIGURE 3. Induction of HIV gp120-specific Th1 immune responses by spleen cells obtained from immunized mice. Spleen cells obtained from BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated with p18-labeled spleen cells, and supernatants were assessed for cytokine concentrations. The results are expressed as the mean concentration ± SE of five mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

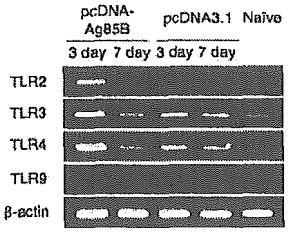

FIGURE 4. TLR mRNA expression profiles of the DNA injection site with electroporation in mice immunized with pcDNA-Ag85B or pcDNA3.1 alone. Total RNA was isolated 3 and 7 days after injection and was analyzed by RT-PCR for TLR2, TLR3, TLR4, and TLR9 mRNA expression. Equality of the RT reaction of isolated RNA between samples was confirmed by amplification of β-actin. Data are representative of three independent experiments.

lytic activities of the cells against p18-I10-pulsed target cells were assessed. Effector cells from mice immunized with pJWNL432 and pcDNA-Ag85B showed higher levels of p18-I10-specific lytic activity than effector cells from BCG-unprimed mice. Moreover, these cytolytic activities were clearly enhanced by BCG sensitization in mice immunized with pJWNL432 mixed with pcDNA-Ag85B (Fig. 5*A*). These effector cells from immunized mice were cultured in a medium containing anti-CD4 or anti-CD8 mAb during the ^{51}Cr release assay. Anti-CD8 mAb inhibited cytolysis against target cells pulsed with the peptide, whereas anti-CD4 mAb did not affect this effector cell function (Fig. 5*B*). Therefore, these results indicate that effector cells expressed CD8 and used this molecule to recognize the target cells. Furthermore, lytic activities of peptide-specific effector cells from immunized mice against MHC-matched or mismatched target cells labeled with the peptide were assessed. These p18-I10-specific effector cells lysed MHC-matched, H-2^d target cells, but not mismatched, H-2^b target cells pulsed with the peptide (Fig. 5*C*). Moreover, the functions of these p18-I10-specific effector cells were inhibited by anti-H-$2D^d$ mAb, but not by anti-H-$2K^d$ mAb or anti-H-$2L^d$ mAb (Fig. 5*D*).

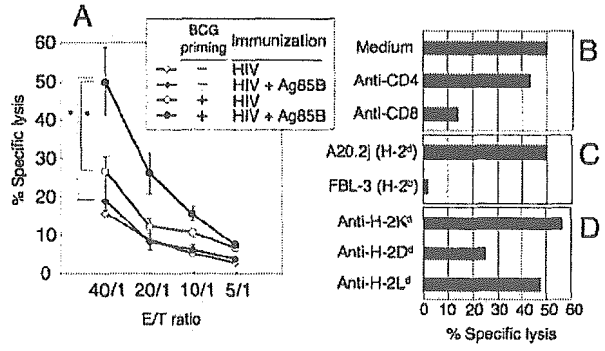

FIGURE 5. Spleen cells from BCG-primed mice coadministered pJWNL432 and pcDNA-Ag85B showed high levels of HIV gp120-specific MHC class I-restricted lytic activity. *A*, $CD8^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B, and the cells were then stimulated with p18-I10-labeled spleen cells and assessed for lytic activities against p18-I10-pulsed target cells. *B*, Lytic activities of effector cells were assessed in the presence of anti-CD4 mAb, anti-CD8 mAb with complement, or medium. The E:T cell ratio was 40:1. *C*, Lytic activities of effector cells against p18-I10-pulsed H-2^d or H-2^b target cells were assessed. The E:T cell ratio was 40:1. *D*, Effector cells were examined for p18-I10-specific lytic activities in the presence of anti-H-$2K^d$, anti-H-$2D^d$, or anti-H-$2L^d$ mAb. The E:T cell ratio was 40:1. Each value is the mean percentage of the specific lysis values obtained from five mice. *, $p < 0.01$.

These results indicated that effector cells elicited in immunized mice were $CD8^+$ and MHC class I-restricted CTLs and suggested that Ag85B has potent adjuvant activities for enhancement of CTL responses by being mixed with DNA vaccine Ag.

Ag85B increase the number of HIV gp120-specific, IFN-γ-secreting, $CD8^+$ T cells

ELISPOT assays were performed to compare the numbers of HIV gp120-specific, IFN-γ-secreting, $CD8^+$ cells in immunized mice. $CD8^+$ T cells purified from spleens of immunized mice were stimulated with peptide-pulsed P815 cells in ELISPOT filter plates coated with an IFN-γ capture mAb for 24 h. The numbers of spots were counted automatically using a KS ELISPOT system. In BCG-unprimed mice, the number of p18-I10-specific IFN-γ-secreting $CD8^+$ T cells was slightly increased in mice coadministered pcDNA-Ag85B (20.3 ± 10.0/10^6 cells) compared with that in mice immunized with pJWNL432 alone (14.0 ± 3.6/10^6 cells). In BCG-primed mice, however, the number of p18-I10-specific IFN-γ-secreting $CD8^+$ T cells was ~3.7-fold greater in mice coadministered pcDNA-Ag85B (96.7 ± 13.3/10^6 cells) than in mice immunized pJWNL432 alone (26.3 ± 5.1/10^6 cells; Fig. 6*A*). To confirm whether the improved CTL responses strictly depend on the presence of Ag85B, BCG-primed mice were coadministered various doses of pcDNA-Ag85B, and the frequency of anti-p18-I10-specific IFN-γ-secreting $CD8^+$ T cells was determined by ELISPOT assay. The number of anti-p18-I10-specific, IFN-γ-secreting, $CD8^+$ T cells was gradually increased by coadministration of Ag85B in a dose-dependent manner (Fig. 6*B*). In addition, dose dependency in improving the anti-p18-I10-specific response was not found in mice coadministered a control plasmid, which expresses an unrelated protein constructed by the same expression vector (data not shown). These results clearly indicate that the

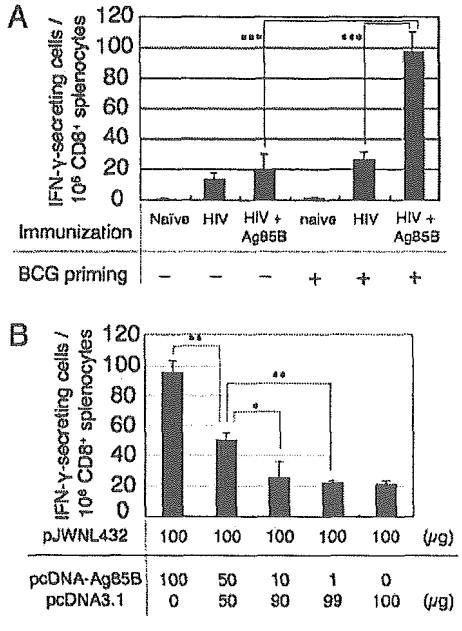

FIGURE 6. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific, IFN-γ-secreting cell frequency. $CD8^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B (*A*) or with various doses of pcDNA-Ag85B (*B*) and were examined for IFN-γ production in an ELISPOT assay after stimulation with p18-I10-labeled P815 cells. Data are presented as the mean number of p18-I10-specific spots per 10^6 $CD8^+$ spleen cells ± SE of five mice per group. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$.

anamnestic response to Ag85B could enhance the simultaneously induced CTL responses. These data also support the results for CTL responses and suggest that coadministration of pcDNA-Ag85B, especially in BCG-primed mice, induces high frequency, Ag-specific, responding $CD8^+$ T cells.

Ag85B enhances protective immunity against rVV-HIVenv infection

Finally, to determine the functional relevance of HIV gp120-specific CTLs generated by immunization with these DNA vaccines to eliminate the virus infection, immunized mice were challenged with 5×10^6 PFU of rVV-HIV gp120. Five days after the challenge, the mice were killed, and the ovaries were harvested and used for estimation of the vaccinia virus titer by RT-PCR, because the ovary is the organ in which the vaccinia virus preferentially replicates. The titers of rVV-HIV gp120 in mice coadministered pcDNA-Ag85B were much lower than those in mice immunized with pJWNL432 alone. Moreover, this inhibitory effect on replication of rVV gp120 was clearly demonstrated in mice primed with BCG before immunization (Fig. 7). These results indicated that immunization of mice with pJWNL432 mixed with pcDNA-Ag85B resulted in the generation of an effector T cell response capable of recognizing endogenously processed viral protein, and that DNA immunization inhibited the replication of rVV-expressing HIV gp120 in vivo.

Discussion

DNA vaccination is a practical and effective way to induce cellular immune responses, especially a CTL response, and has shown great promise for initiating cellular immune responses that are regulated for the prevention of various disease such as tumors, HIV, tuberculosis, hepatitis C virus, and malaria. In humans and large animal models, however, immune responses induced by DNA vaccination are not sufficient for prevention or control of infection. Thus, there is a need to increase the potency of DNA vaccines for use in humans. In the present study we investigated the effectiveness of a novel approach for enhancing the ability of a DNA vaccine to induce cellular immune responses by using previously experienced immunogenic proteins that induce a strong Th1-dominant immune response.

$CD4^+$ T cells play a critical role in the generation and maintenance of CTL responses in a noninflammable vaccination strategy including conventional plasmid DNA vaccination. Convincing evidence that dendritic cells (DCs) are the principal cells for priming

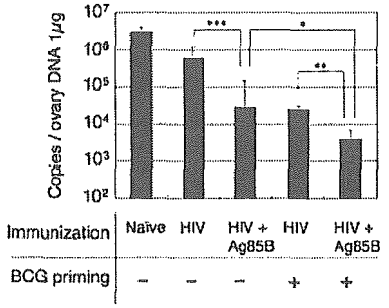

FIGURE 7. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific protective immunity. BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were challenged with 5×10^7 PFU of rVV-HIV-gp120. The bars show the virus as the log of the number of virus copies in ovaries of mice. The data represent the mean copies of virus obtained from five mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

$CD8^+$ T cells in DNA vaccination through direct transfection of DNA (19–21) or cross-presentation of a vaccine-derived Ag has been presented (22–24). Activation of $CD4^+$ T cells is required for maturation of DCs through CD40 signaling (3–5); however, this requirement is not sufficient in many cases of DNA vaccine immunization (25, 26). Various studies on compensation for the insufficiency of $CD4^+$ T cell help by coadministration of costimulatory molecules, such as B7-1 or B7-2 (27, 28), or by treatment with a CD40 agonist during immunization have therefore been conducted (26). Another $CD4^+$ T cell-mediated helper effect for induction of CTL by DNA vaccine is thought to be the production of cytokines for enhancement of activity and proliferation of CTLs (29). Cognate $CD4^+$ T cell help is important for inducing pathogen-specific CTLs (30), and cognate $CD4^+$ T cell help should be induced to elicit HIVgp120-specific CTLs by injection of DNA vaccine. The Ag85B in our system enhances this cognate manner and polarizes Th1-type immune responses as a vaccine adjuvant. Numerous studies have focused on the effectiveness of coadministration of Th1 cytokine-encoding plasmids, such as IL-2, IL-12, IL-15, IL-18, IL-23, and IFN-γ, for enhancing CTL responses (31, 32). However, adjuvant effects of cytokines on CTL generation are different (33, 34), suggesting that it is necessary to augment the immune responses by these strategies for administration not only of the combination of cytokines codelivered, but also for the timing of administration (35, 36). The expression of both Ag85B and HIV Ag in the same tissues provides this advantage, because Ag85B is thought to be a strong potentiator of Th1-type cytokines. In fact, our results showed production of IFN-γ from spleen cells after stimulation with HIV Ag (Fig. 3).

BCG is an important clinical tool because of its strong immunostimulatory properties. Humans as well as resistant mouse strains infected with BCG predominantly exhibit a Th1 cytokine secretion profile (37). Although specific Ags eliciting Th1 cell responses in mycobacteria are not yet known, a recent study suggested that one of the immunogenic proteins for Th1 development is Ag85B (9). Apparently, strong Th1 responses have been elicited in vitro from purified protein derivative-positive asymptomatic individuals using purified Ag85B (38–40). Furthermore, vaccination of mice with plasmid DNA encoding Ag85B induced strong Ag85B-specific CD4 T cell proliferation and vigorous IFN-γ secretion, resulting in the protection of further *Mycobacterium tuberculosis* infection (41). We have also shown that Ag85B-specific recall responses enhance tumor-specific cellular immune responses in Ag85B gene-transfected tumor cell immunization (11). One possible reason for Th1 domination by Ag85B is that the immunogenic Th1-inducing helper epitope, known as peptide-25, is included in Ag85B protein (42, 43). Peptide-25 was able to stimulate proliferation and a high amount of IFN-γ production in *M. tuberculosis*-primed cells (42). It remains unclear why peptide-25 can induce potent Th1 responses; however, several recent studies have suggested that the avidity of the peptide for its specific TCR may be strong enough to induce Th1 development (9, 44). It is now generally accepted that MHC class II-dependent activation of $CD4^+$ T cells, mainly Th1-polarized cells, potently enhances concomitantly existing unrelated CTL responses (7, 44). According to this line of reasoning, coadministration of Ag85B DNA is a promising tool for enhancement of CTL responses through Ag85B-specific Th cell proliferation and Th1 polarization in a DNA vaccination strategy.

The roles of some proteins and peptides in the polarized development of Th1 cells have been reported, and Ag85B is considered to be one such protein. In fact, we found therapeutic effects of Ag85B on Th2-type allergic disease, asthma, and atopic dermatitis (unpublished observations). The mechanisms, however, are still

not clear. Various products with adjuvant activities, such as LPS, CpG motif, or polyinosinic-polycytidylic acid, involve TLRs and show augmentation of Th1-type immune responses (18). Bacterial components, mycobacterial lipoprotein, bacterial peptidoglycan, and flagellin, also associate with TLRs (18). A correlation between the adjuvant activities of Ag85B and TLRs has not been found. Mycobacteria can bind some TLRs and show typical Th1-type immune responses (45). In a transfection model using Chinese hamster ovary cells (which are relatively deficient in TLRs), the expression of TLR2 or TLR4 conferred responsiveness to both virulent and attenuated *M. tuberculosis* (46). Lipoarabinomannan, a major mycobacterial cell wall component, appears to resemble the cell wall component of Gram-negative bacterial LPS. TLR2 was shown to be necessary for signaling of mycobacterial LPS lipoarabinomannan (47). An undefined, heat-labile, cell-associated, mycobacterial factor was found to be the ligand for TLR4 (47). Ag85B might be included in one of these factors, if it is involved in innate immunity through TLRs. In fact, our results showed enhancement of the expression of TLR2, TLR3, and TLR4 in Ag85B DNA-injected mice (Fig. 4). Because it has been reported that not only microbial components, but also several cytokines regulate the expression of TLRs, there is the possibility of secondary responses for the expression of TLRs by induction of cytokine (18).

Another important biological role of Ag85B is binding of fibronectins (FNs) (48–50). FNs are a family of high molecular weight glycoproteins found in plasma and tissues and are involved in cell motility and adhesion, regulation of cell morphology, phagocytic function, and wound healing (51). Many integrin-binding sites have been identified in amino acid sequences of FNs (52), and adhesion of FN-binding proteins to FNs helps the phagocytosis of proteins into integrin-expressing APCs, especially monocytes, macrophages, and DCs (53). Binding of FNs to human monocytes enhances the phagocytic function of monocytes for bacilli (51), and inhibition of FN-integrin receptor interaction can prevent *M. kansasii* phagocytosis (54). Moreover, Ag85B from *M. tuberculosis* and FNs synergistically stimulate TNF-α expression in human monocytes (55), suggesting that the binding ability of Ag85B with FNs influences not only the enhancement of incorporation of Ags into phagocytic cells, but also the construction of the Th1 milieu at the site of injection.

The results of the present study suggest that coadministration of Ag85B DNA has several potential advantages over other genetic adjuvants due to the existence of multiple mechanisms for elicitation of CTL responses by a DNA vaccine. The results also showed the effectiveness of mycobacterial sensitization for enhancing adjuvanticity of Ag85B. Because most humans have been sensitized by spontaneous infection or by vaccination with mycobacteria, this finding is valuable for the possible use of Ag85B as a genetic adjuvant of a DNA vaccine. The results of this study have provided evidence of the potential utility of Ag85B for the development of a DNA vaccination strategy for successful human use.

Disclosures

The authors have no financial conflict of interest.

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Sequence Note

Molecular Epidemiology of the Heterosexual HIV-1 Transmission in Kunming, Yunnan Province of China Suggests Origin from the Local IDU Epidemic

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ABSTRACT

Molecular epidemiological investigation was conducted among injecting drug users (IDUs) ($n = 11$) and heterosexuals ($n = 15$) in Kunming, Yunnan Province of China. HIV-1 genotypes were determined based on the nucleotide sequences of 2.6-kb *gag*-RT region. The distribution of genotypes among IDUs was as follows: CRF07_BC (5/11) and CRF08_BC (5/11); subtype B′ (1/11). Similarly, a majority of Kunming heterosexuals (14/15) were infected with CRF07_BC (4/15), CRF08_BC (6 /15), or subtype B′ (4/15), known to predominate among IDUs in China. This contrasts with trends in the coastal regions of China and surrounding southeastern Asian countries, where CRF01_AE predominates among heterosexuals. The heterosexual HIV-1 epidemic in Kunming thus appears to derive from the local IDU epidemic. Of note, subtype B′ was the most prevalent strain among heterosexuals before 1997, while CRF07_BC and CRF08_BC became predominant in 2002, indicating a transition of HIV-1 genotype distribution between the early and the more recent samples from Kunming heterosexuals.

THE HIV-1 EPIDEMIC IN CHINA was first detected among injecting drug users (IDUs) in the western part of Yunnan Province in 1989. HIV prevalence among IDUs in initial epidemic sites reached 50–80% by 1993.[1] Yunnan Province accounted for more than 80% of the HIV-1 infections reported in China through 1996[1] and is thought to be an epicenter of the HIV epidemic in China. According to recent HIV-1 sentinel surveys, the HIV-1 prevalence rate among newly tested IDUs in Yunnan has been stable (19.7–24.7% in 1997–1999).[2] However, HIV-1 prevalence rates among female commercial sex workers (CSWs) and wives of heroin users have increased steadily. For example, HIV prevalence among CSWs in Yunnan increased from 1.0% in 1997 to 3.4% in 2001.[2] Figure 1 shows the study site and the geographical distribution of the numbers of HIV reported cases in China as of June 2003 (http://www.aids.net.cn).

HIV-1 strains circulating in Yunnan showed extremely high genetic diversity. Various HIV-1 strains, including subtypes B, B′[3] (Thailand variant of subtype B, also referred to as Thai-B[4]) and C,[5,6] and CRF07_BC and CRF08_BC[7]), have been detected among IDUs. Moreover, in addition to these HIV-1 strains, diverse forms of unique recombinants between subtypes B′ and

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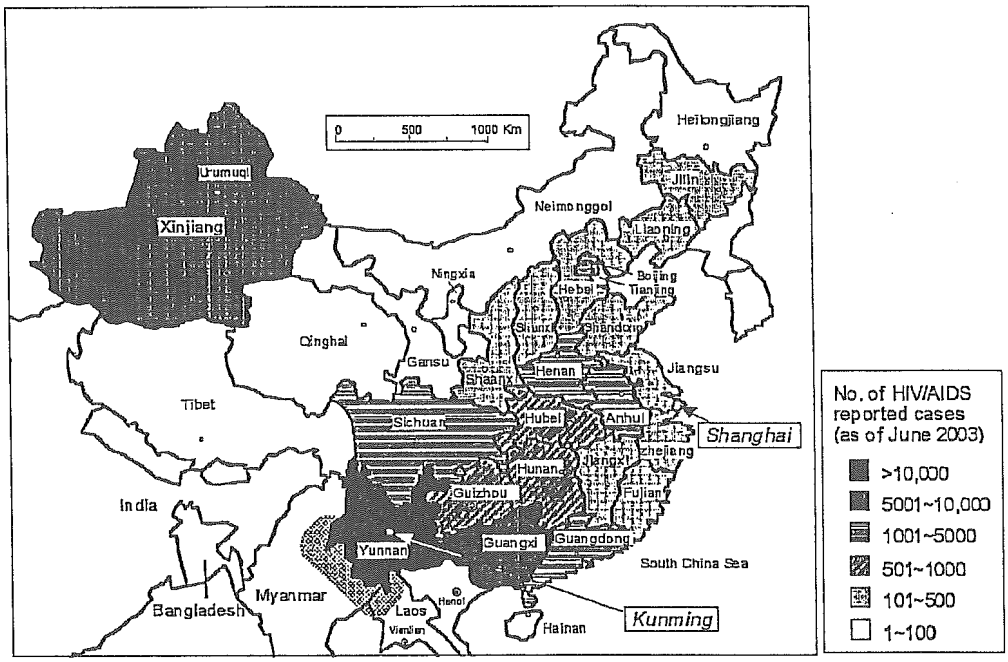

FIG. 1. Map of China. The study site (Kunming, Yunnan Province) and the geographical distribution of the numbers of HIV reported cases (as of June 2003) are shown (http://www.aids.net.cn). The so-called "Golden Triangle," a major heroin production, refining, and trading area, at the borders of Thailand, Myanmar, and Laos, near Yunnan Province, is marked.

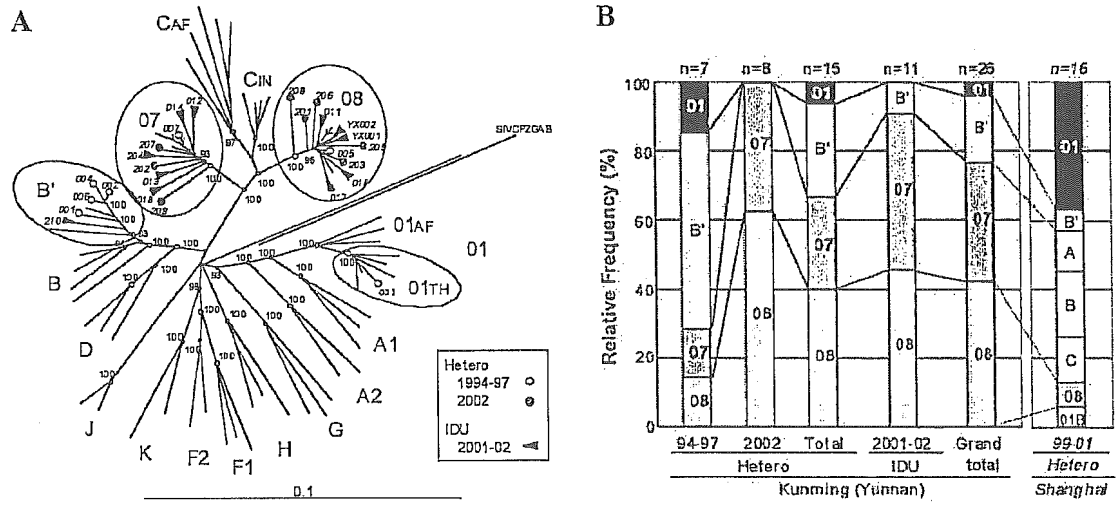

FIG. 2. Phylogenetic tree analysis and the distribution of HIV-1 strains circulating among different risk populations in Kunming, Yunnan. (A) Neighbor-joining tree based on the nucleotide sequences of 2.6-kb *gag*-RT regions with HIV-1 group M reference strains (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html). $SIV_{CPZ}GAB$ was used as an outgroup. Bootstrap values (>90) are shown at the corresponding nodes. Subtype and CRF designations are shown outside the tree. HIV-1 specimens from IDUs were collected in 2001–2002 (closed arrowheads); HIV-1 specimens from heterosexuals sampled in 1994–1997 (open circles) and in 2002 (striped circles). Three-digit numbers indicate the specimen codes. (B) Distribution of HIV-1 genotypes in different risk populations. Bars indicate the relative frequency (%) of the indicated HIV-1 genotype in the respective sample category shown at the bottom. The data on HIV-1 genotype distribution among heterosexuals in Shanghai are adopted from Zhong *et al.*[15] *n* indicates the number of the specimens analyzed in each sample category shown below. B′, HIV-1 subtype B′ (Thailand variant of subtype B); C, subtype C; 01, CRF01_AE; 07, CRF07_BC; 08, CRF08_BC; C_{AF}, African subtype C; C_{IN}, Indian subtype C; 01_{AF}, African CRF01_AE; 01_{TH}, Thailand CRF01_AE.

C,[7] and even the second-generation recombinants comprised of CRF07_BC and CRF08_BC,[8] have been reported among IDUs in Yunnan. In contrast, however, information on the HIV-1 genotypes circulating among heterosexuals in Yunnan is very limited. Although an early study detected CRF01_AE in women who had returned from commercial sex work in Thailand[9] and HIV-1 subtype B/B′, C, CRF01_AE, and CRF08_BC have recently been reported in a small number of heterosexuals in Yunnan,[2] HIV-1 genotypes circulating among persons at heterosexual risk have not been well studied. Ongoing monitoring of the HIV-1 genotype distribution in Yunnan would be important for understanding the evolution of the epidemic as well as for future vaccine strategies in China.

To track the HIV-1 genotype distribution in Yunnan, we collected a total of 26 HIV-1-positive plasma samples from persons in the capital city of Kunming and environs during 1994–2002. Fifteen specimens were from persons who acquired HIV-1 infection through heterosexual contact (7 were sampled in 1994–1997 and 8 were collected in 2002). Eleven specimens were collected from IDUs in 2001–2002. The nucleotide sequences of HIV-1 *gag*-RT regions (2.6 kb) were determined on both strands using BigDye terminator reaction kits on an ABI 373 DNA sequencer as described previously.[7] A multiple alignment with HIV-1 group M references (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html) was generated by the Se-Al program.[10] HIV-1 genotypes were screened and determined based on phylogenetic tree (Fig. 2A) and recombination breakpoint analyses of *gag*-RT regions. Phylogenetic trees were constructed by the neighbor-joining method[11] using PHYLIP package version 3.6a3[12] and the reliability of topologies of trees was tested by bootstrap analysis with 100 bootstrap replicates.[13] Bootscanning analyses were performed on neighbor-joining trees for a window of 200 bp moving along the alignment in 30-bp increments, using the Simplot program.[14]

The distribution of HIV-1 genotypes in a total of 26 samples is as follows (Fig. 2): HIV-1 subtype B′ (Thailand variant of subtype B) (5, 19%); CRF01_AE (1, 4%); CRF07_BC (9, 35%); and CRF08_BC (11, 42%). As shown in Fig. 2B, CRF07_BC (5 of 11, 45%) and CRF08_BC (5 of 11, 45%) are predominantly distributed among IDUs. In contrast, HIV-1 subtype B′ (4 of 7, 57%) was the most common strain among specimens from heterosexuals before 1997, while CRF01_AE, CRF07_BC, and CRF08_BC occurred only infrequently (1 of 7, 14% each). Interestingly, however, CRF07_BC (3 of 8, 38%) and CRF08_BC (5 of 8, 63%) were more common among specimens collected from heterosexuals in 2002, indicating a transition of HIV-1 genotype distribution between the early (before 1997) and the more recent samples (in 2002) from Kunming heterosexuals.

It is noted that the specimens, 208 (02CNKM208) and 209 (02CNKM209), are placed slightly outside the clusters of CRF08_BC and CRF07_BC, respectively (Fig. 2A). The raw direct sequencing data of theses specimens contained several ambiguous signals. The clonal sequence analysis by TA cloning revealed that they were coinfected with another lineage of the HIV-1 strain (X.-J. Li, in preparation).

The small proportion of CRF01_AE among heterosexuals in Kunming (Fig. 2) contrasts with the findings in surrounding Southeast Asian countries, where CRF01_AE shows a strong founder effect triggering the explosive epidemic among heterosexuals.[5] As shown in Fig. 2, it appears that CRF01_AE has not accounted for the majority of sexual transmission in Kunming. Although CRF01_AE was detected in the early 1990s among returnees from Thailand,[9] it has not gained the momentum of dissemination through the sexual route in Kunming, as it has in other Southeast Asian countries. In contrast, CRF01_AE constituted a significant proportion of HIV-1 strains among heterosexuals (6 of 16, 38%) in the city of Shanghai in 1999–2001[15] (Fig.2B. right). This suggests a difference in the structure and the genesis of heterosexual epidemics in Kunming and the coastal areas represented by Shanghai. Heterosexual transmission of HIV-1 in Kunming thus appears to be strongly influenced by the local IDU epidemic, while CRF01_AE shows a significant founder effect among heterosexuals in some coastal regions in China.

In conclusion, the apparent predominance of CRF07_BC and CRF08_BC among heterosexuals in Kunming suggests that a large proportion of these infections are related to IDU networks in China. These findings would contribute to our understanding of the HIV-1 epidemic in China.

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Patterns of Point Mutations Associated With Antiretroviral Drug Treatment Failure in CRF01_AE (Subtype E) Infection Differ From Subtype B Infection

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Summary: An increasing number of HIV-1–infected patients living in developing countries now have access to antiretroviral drugs. Information regarding the drug-resistant mutations of non-B subtype HIV-1 remains limited, however. The authors cross-sectionally compared patterns of the drug-resistant point mutations in patients infected with either subtype B or CRF01_AE (subtype E) among patients who acquired HIV by sexual transmission in Japan. Protease sequence data were available from 216 patients with a detectable level of RNA copies in plasma. Based on phylogenetic analysis of the protease and the C2V3 regions, 162 subtype B and 45 CRF01_AE cases were identified; 82 subtype B and 24 CRF01_AE patients had a treatment failure with nucleoside reverse transcriptase inhibitors; and 69 subtype B and 19 CRF01_AE patients had a treatment failure with a protease inhibitor. Antiretroviral drug history was similar in subtype B-infected and CRF01_AE-infected patients. The mutations T69N and V75M in reverse transcriptase and L10F, K20I, L33I, and N88S in protease were seen more frequently in patients infected with CRF01_AE than in patients with subtype B. The mutations, D30N, A71V, and N88D were found exclusively in patients with subtype B. Most of the characteristic mutation patterns were associated with a history of receiving nelfinavir. The pattern of drug resistance mutations differs between the subtypes. Data derived from subtype B drug-resistant genotypes may not always be applicable to non-B subtypes. **Key Words:** HIV, antiretroviral drug, CRF01_AE, drug resistance, genotype

An increasing number of HIV-1–infected patients living in developing countries now have access to antiretroviral drugs for the prevention of mother-to-child HIV-1 transmission and for improving the quality and length of patients' lives as prices of antiretroviral drugs rapidly fall. The majority of patients living in such countries were infected with non-B subtype HIV-1. In Japan, the proportion of heterosexually transmitted patients has been rising, and we have previously reported that the non-B subtype, particularly CRF01_AE (subtype E), is becoming common among the heterosexually infected population.[1]

Amino acid sequence diversity in the *pol* gene is 10% to 15% between subtypes.[2] Even a single amino acid mutation can dramatically change the susceptibility or the resistance of the virus to an antiretroviral drug.[3,4] Thus, given this genetic diversity, it is plausible that non-B HIV-1 subtypes evolve differently from B subtypes, and this may be reflected in different patterns and pathways of resistance to antiretroviral drugs. Studies on antiretroviral drug resistance of non-B subtypes have been limited, however, in comparison with studies done on subtype B. We believe that it will be important to have more data on non-B subtype drug resistance mutations to monitor the inevitable emergence of drug-resistant HIV-1 in these countries and to optimize anti-

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retroviral drug treatment of patients infected with non-B subtype. In this article, we show results of *pol* sequence analysis among drug-naive and antiretroviral-treated patients infected with CRF01_AE in Japan in comparison with subtype B–infected patients. Some patterns of drug resistance mutations in patients infected with CRF01_AE, who were clinically resistant to antiretroviral drug therapy, were different from those observed in patients infected with subtype B.

MATERIALS AND METHODS

Study Design and Sample Selection

Since November 1996, Japan's AIDS Research Center, National Institute of Infectious Diseases, has been providing a genotyping service for clinicians in referral hospitals throughout the country. A retrospective cross-sectional study was conducted to analyze the pattern of drug resistance-associated mutations among all samples that we have received from sexually transmitted HIV-1–positive patients between November 11, 1996 and September 7, 2000.

The patients were first stratified clinically according to their viral load reduction in response to drug therapy. If the viral load was reduced to less than 400 copies/mL within 3 months of antiretroviral therapy, the patients were classified as drug sensitive. If the viral load remained more than more than 1000 copies/mL after 3 months of antiretroviral therapy, the patients were classified as having treatment failure. Patients were unclassified if they had a viral load more than 400 copies/mL and less than 1000 copies/mL on antiretroviral therapy, if they were on antiretroviral drugs for less than 3 months of therapy, or if they had taken antiretroviral therapy but were off drugs at the time of sample collection. If multiple samples existed from 1 patient, only the most recent sample was analyzed. There were 27 cases in which patients responded to a salvage therapy, including nonnucleoside reverse transcriptase inhibitors (NNRTIs). In these cases, we selected the last sample before the start of salvage therapy. Patients who had not had antiretroviral drugs prior to sample collection were regarded as drug naïve. These samples were included for cross-sectional observation to analyze baseline amino acid residues at drug resistance-associated sites. If multiple samples were available from drug-naive patients, we selected the earliest samples before therapy. There was no overlap between patients with drug treatment failure and drug-naive patients. The sample selection was independent of subsequent sequence analysis.

Sequencing Method

Sequencing HIV-1 reverse transcriptase (RT) and protease regions has been undertaken in accordance with a previously published method.[5] Briefly, viral RNA was extracted from 200 μL of plasma with an RNA extraction kit (High Pure Viral RNA Kit; Boehringer Mannheim GmbH, Mannheim, Germany); a 464-base pair (bp) protease fragment (base number of nucleotide: 2148–2611) and an 888-bp RT fragment (2485–3372) were separately amplified by PCR after a reverse transcription reaction from extracted RNA by means of an RNA-PCR kit (One Step RNA PCR Kit [AMV]; TaKaRa, Osaka, Japan). Primary PCR products were further amplified with a high-fidelity DNA polymerase (KOD DNA Polymerase; TOYOBO, Osaka, Japan).

For C2/V3 subtyping, DNA was extracted from peripheral blood mononuclear cells (PBMCs) with a DNA extraction kit (QIAamp DNA Blood Mini Kit; QUIAGEN, Germany) and a 380-bp C2/V3 fragment (7001–7380) was amplified with nested PCR using a high-fidelity PCR system (Expand High Fidelity PCR System, Boehringer Mannheim). Sequencing analysis was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA) and an autosequencer (ABI PRISM 377 DNA Sequencer; Applied Biosystems). Sequence results were then analyzed by computer software (Sequence Navigator version 1.0.1, Applied Biosystems). For protease and RT drug resistance genotyping, sequence results were compared with the HXB2 reference sequence and mutation points were determined. Subtypes were determined by phylogenetic analysis; C2V3 and protease sequence results were aligned by means of the clustal-W program with a set of reference sequences recommended by the Los Alamos sequence database. The results of the alignment were then analyzed by the neighbor-joining method. This analysis also confirmed that each sequence was unique; the possibility of sequence contamination was ruled out.

Statistical Method

Proportions of mutations at each codon were first compared between drug-naive subtype B and CRF01_AE patients and between subtype B and CRF01_AE patients with treatment failure by the χ^2 test. If any significant difference was noted at $p < .05$, we compared proportions of an individual amino acid substitution with the χ^2 test or Fisher exact test if an expected cell value was less than 5. The Mantel-Haenszel method was applied to adjust for the pattern of drug usage; if a patient had a drug for at least 3 months, the patient was regarded as being significantly exposed to the drug. Continuous data were analyzed using a nonparametric test, the Kruskal-Wallis (Wilcoxon) test. The analysis was conducted using Epi Info version 6.04.

RESULTS

During the study period, we received 745 samples from 261 sexually transmitted HIV-1–positive patients from 25 referral hospitals: of these, 393 samples were taken from 115 heterosexual patients, 307 samples from 127 homosexual patients, and 45 samples from 19 patients with unknown sexual behavior. Single samples were received from 112 patients, whereas multiple samples were received from 149 patients, where the median (interquartile range [IQR]) number of samples per patient was 3,[2,5] ranging up to 35 samples per patient.

Subtyping

Protease sequence data were available from 216 patients with a detectable level of RNA copies in plasma and subjected to phylogenetic analysis for determining subtype. Protease sequence was not available in 45 patients; this group was characterized by a low level of viral load and a high CD4 count: 74% had an undetectable viral load (<50 copies/mL), the median (IQR) CD4 count was 391 (222, 612) cells/μL, and most patients (96%) had received antiretroviral drugs. One hundred

sixty-two of 216 (75.0%) patients had subtype B sequences in the protease region, 45 of 216 (20.8%) patients had CRF01_AE, 3 of 216 (1.4%) patients had F subtype, 1 of 216 patients had C subtype, 1 of 216 patients D subtype, and 4 of 216 (1.9%) patients had an unknown subtype. Sequencing of the V3 region was performed in 34 protease subtype B and 27 protease subtype CRF01_AE samples. All patients with subtype B or CRF01_AE in the protease region had concordant subtypes in the V3 region. This result supported an assumption that subtype CRF01_AE can be differentiated from subtype B on the basis of phylogenetic analysis of the protease region in this study population.

Comparison of Subtype B and CRF01_AE Patients

Table 1 summarizes sexual behavior, sex, age, and clinical response to antiretroviral drugs in 162 subtype B and 45 subtype CRF01_AE patients. The proportion of male patients was significantly higher in subtype B patients than in CRF01_AE patients ($p < .0001$). The median age was similar in both groups. Sexual behavior was reported in 191 patients; CRF01_AE infection was strongly associated with a heterosexual route of acquisition ($p < .0001$). The trend for median viral load to be higher in subtype B patients than in CRF01_AE patients was seen but not significant. The median CD4 count was significantly lower in CRF01_AE patients than in subtype B patients ($p = .0001$), and the proportion of AIDS cases (C1, C2, and C3) was significantly higher in CRF01_AE patients ($p = .038$). Patients were classified according to their history of taking antiretroviral drugs and their viral load profiles. The distribution of clinical responses to nucleoside reverse transcriptase inhibitors (NRTIs) or protease inhibitors (PIs) among CRF01_AE patients was similar to that among subtype B patients.

We further compared the history of NRTI use in 86 subtype B and 24 CRF01_AE patients with NRTI drug treatment failure (Table 2*A*). There were no significant differences in the history of NRTI regimens in terms of drug selection or median duration in months on NRTI treatment. We also compared the history of PI use in 68 subtype B and 19 CRF01_AE patients with PI drug treatment failure (see Table 2*B*). There were similarly no significant differences in the history of PI regimen in terms of drug selection or median duration in months on PI treatment.

TABLE 1. *Background information of patients*

	Subtype B	CRF01_AE	Total (N = 207)
Sex			
Male	149	30	179
Female	13	15	28
Age Median (range) years	36.5 (21, 78)	36.0 (21, 62)	36.0 (21, 78)
Sexual behavior			
Heterosexual	45	40	85
Homosexual	105	1	106
Unknown	12	4	16
RNA copies/mL			
Median (IQR)	17,050 (2400)	36,300 (5900)	23,200 (3000)
CD4 count[a]			
Median (IQR)	294 (158.5, 432)	115 (39, 317)	264 (100, 393)
Proportion of AIDS (C1, C2, and C3)[b]	38/148 (25.7%)	18/41 (43.9%)	56/189 (29.6%)
Response to NRTI			
Failure	86 (53%)	24 (53.3%)	110
NRTI naive	36 (22.2%)	8 (17.8%)	44
Sensitive	13 (8%)	5 (11.1%)	18
Unclassified	23 (14.2%)	5 (11.1%)	28
Data not available	4 (2.5%)	3 (6.7%)	7
Response to PI			
Failure	68 (42%)	19 (40%)	87
PI naive	59 (36.4%)	14 (33.3%)	73
Sensitive	11 (6.8%)	4 (8.9%)	15
Unclassified	20 (12.3%)	5 (11.1%)	25
Data not available	4 (2.5%)	3 (6.7%)	7

IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
[a] $p = .0001$.
[b] $p = .038$.

TABLE 2. *Drug history of patients with drug treatment failure*

A. Nucleoside reverse transcriptase inhibitory history	Subtype B (N = 86)	n	CRF01_AE (N = 24)	n
AZT	23.5 (14, 34)	44	29 (14, 36)	14
Off AZT	15 (8, 26)	41	21 (10, 26)	9
3TC	21 (9, 31)	51	24 (17, 34)	17
Off 3TC	10 (6, 16)	26	9 (1, 9)	6
D4T	16 (12, 21)	43	22.5 (17, 27)	10
Off d4T	8 (5, 12)	9	9.5 (5, 14)	2
ddC	22 (18, 27)	7	12	2
Off ddC	9.5 (6, 17)	14	12 (4, 18)	3
ddI	16 (7, 24)	25	19 (12, 30)	5
Off ddI	9 (6, 12)	22	8 (3, 13)	8
Nevirapine	4.5	2	19	1
Off nevirapine	14	1	—	—
Efavirenz	—	—	3	1

B. Protease inhibitor history	Subtype B (N = 68)	n	CRF01_AE (N = 19)	n
Nelfinavir	16 (10, 22)	42	20 (12, 27)	11
Off nelfinavir	8 (5, 12.5)	8	5 (1, 10)	4
Indinavir	25 (12, 29)	14	15.5 (12.5, 23.5)	4
Off indinavir	12.5 (5.5, 14)	16	9 (3, 12)	7
Saquinavir	11 (8, 15)	7	18 (13.5, 22.5)	4
Off saquinavir	6 (4, 13)	9	6 (5, 6)	3
Ritonavir	11 (8.5, 14.5)	8	12	1
Off ritonavir	5 (1, 12)	7	—	—

The proportions of patients who were receiving each drug at the time of blood sampling (upper row) and the proportion of patients who had been exposed but were not receiving the drug at the time of blood sampling (lower row) are shown. Median (interquartile range) durations in months of the exposure to each drug are also shown.

AZT, azidothymidine; 3TC, lamivudine; D4T, stavudine, ddC, didoxycytidine; ddI, didanosine.

Comparison of *pol* Gene Sequences Among Drug-Naive Patients

We first compared frequencies of amino acid residues in the RT region of 34 NRTI-naive subtype B patients and 8 NRTI-naive CRF01_AE patients. Figure 1 shows amino acid residues at 16 sites where a significant difference was noted. None of the 16 sites was known as an NRTI drug resistance–associated site.[6]

A significant difference in frequencies of amino acid residues was noticed between 57 subtype B and 14 CRF01_AE PI-naive patients at nine sites (Fig. 2), where three sites were known as PI drug resistance-associated sites.[6]

Comparison of Drug Resistance–Associated Mutations Among Patients With Drug Treatment Failure

We then compared the frequencies of amino acid residues among patients with treatment failure between subtypes B and CRF01_AE. Figure 1 shows the frequencies of amino acid residues among the patients with NRTI drug treatment failure. Most of the significant amino acid variations found in drug-naive patients remained significant in the patients with NRTI treatment failure, except for two positions: I31 and G196. The frequency of Gly-to-Glu mutation at position 196 was higher in patients with NRTI treatment failure than in drug-naive patients with CRF01_AE infection, but the trend was the opposite in subtype B infections. Figure 1 also shows the frequencies of amino acid residues at the known NRTI resistance-associated sites. There were no significant differences in the frequencies of amino acid mutations between patients with subtype B and subtype CRF01_AE, except the Thr-to-Asn mutation at position 69 (T69N) and the Val-to-Met mutation at position 75 (V75M) were seen more commonly in CRF01_AE than in subtype B. This difference remained significant even after adjusting for the pattern of NRTI usage, although the difference was marginal. The relation between these mutations and a history of at least 3 months of a specific NRTI was analyzed; no specific drug was significantly associated with T69N; however, the V75M mutation was found to be significantly associated with stavudine (d4T) treatment in CRF01_AE patients ($p = 0.02$) but not in subtype B patients.

Figure 2 summarizes the frequencies of amino acid residues in the protease region among patients with PI treatment failure. It also shows the frequencies of mutations in the amino acid residues at the nine sites where significant differences were found in the drug-naive patients. The significant difference in the frequency of the Ile-to-Leu mutation at position 93 (I93L), which was detected in PI-naive patients, disappeared in PI-resistant patients, whereas the rest of the amino acid variations remained significant. Figure 2 shows the frequencies of mutations in amino acid residues at the other known sites associated with PI resistance; differences in the frequencies of the six amino acid mutations at five sites were detected in PI-resistant patients: Leu-to-Phe at position 10 (L10F), Lys-to-Ile at position 20 (K20I), Leu-to-Ile at position 33 (L33I), and Asn-to-Ser at position 88 (N88S) were more commonly seen in CRF01_AE infection than in subtype B infection. Conversely, the Asp-to-Asn at position 30 (D30N), Ala-to-Val at position 71 (A71V), and Asn-to-Asp at position 88 (N88D) were exclusively seen in subtype B infection. These differences remained significant even after adjusting for the pattern of PI usage. Analysis of specific drug use showed that six mutations (L10F, D30N, L33I, A71V, N88S, and N88D) were significantly associated with a history of nelfinavir therapy but that the K20I mutation was not associated

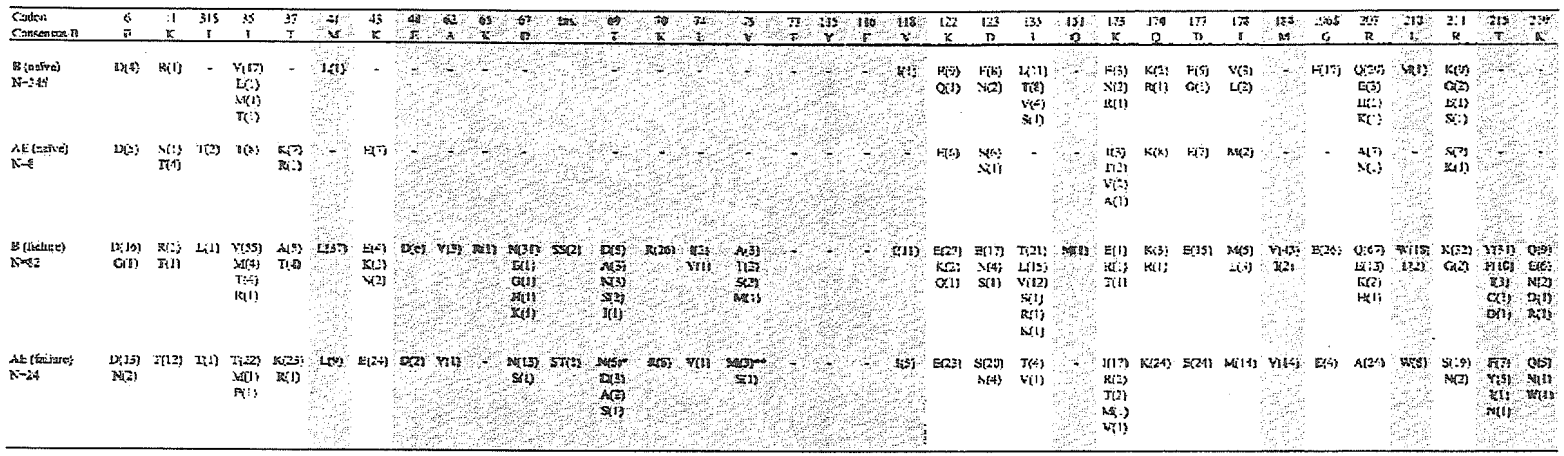

FIGURE 1. Amino acid variations in the reverse transcriptase (RT) region. This figure shows amino acid variation at the known nucleoside reverse transcriptase inhibitor (NRTI) resistance-associated sites (shaded) and other amino acid substitutions, where a significant difference was noted among the NRTI-naive group between subtype B and CRF01_AE ($p < .05$). The frequency of each substitution is shown in brackets. #RT sequence was not available in 2 patients with subtype B. The difference was not significant among NRTI treatment failure group. Ins, insertion. Shown in bold is the site where the frequency remained significantly different among the treatment failure groups between subtype B and CRF01_AE after adjusting for NRTI drug usage patterns (*$p = .04$, **$p = .03$).

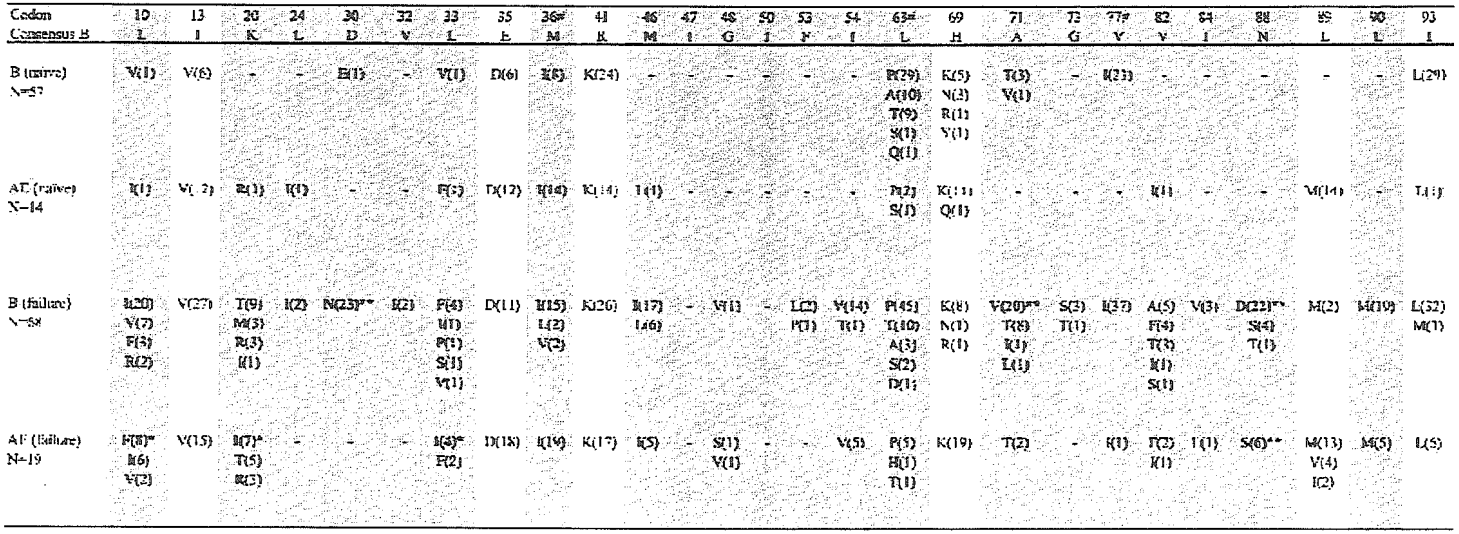

FIGURE 2. Amino acid variation in the protease region. This figure shows amino acid variation at the known protease inhibitor (PI) resistance-associated sites (shaded) and other amino acid substitutions, where a significant difference was noted among the PI-naive group between subtype B and CRF01_AE ($p < .01$). #PI resistance-associated sites where a significant difference was noted among the PI-naive group ($p < .01$). Shown in bold is the site where the frequency remained significantly different among the treatment failure groups between subtype B and CRF01_AE after adjusting for PI drug usage patterns (*$p < .001$, **$p = .01$).

with the use of any particular drug. Interestingly, the A71V mutation was significantly associated with nelfinavir therapy in CRF01_AE patients ($p = .005$), but it was associated with indinavir therapy in subtype B patients ($p = .05$). Similarly, the N88S mutation was significantly associated with nelfinavir therapy in CRF01_AE patients ($p = .004$), whereas this mutation was significantly associated with indinavir therapy in subtype B patients ($p = .002$).

DISCUSSION

Studies have shown that naturally occurring polymorphisms in drug-naive HIV-1–positive individuals are also sites associated with resistance to antiretroviral drugs. These studies found that the RT and the protease sequences of the non-B subtype viruses were highly diverse. Amino acid sequences at positions of known NRTI resistance mutations in subtype B viruses are highly conserved between different subtypes.[7–10] Conversely, amino acids at positions of known PI resistance in subtype B viruses are highly variable in different subtypes.[10,11] The drug susceptibility of non-B subtype viruses has also been studied in phenotypic assays, but the susceptibility to various NRTIs and PIs appears to be similar across the different subtypes.[12,13] Likewise, clinical responses to antiretroviral drug therapy were found to be similar in patients infected with different subtypes.[14] Little has been reported about the characteristics of drug resistance mutation patterns of non-B subtype viruses among patients who do not respond to antiretroviral drug therapy, however. Recently, it has been reported that the prevalence of known drug resistance mutations significantly differs between subtypes B and C.[15] To our knowledge, this is the first report showing that the patterns of drug resistance mutations of CRF01_AE patients also significantly differ from those of subtype B patients.

Our observations show that many amino acid substitutions commonly seen in CRF01_AE patients with drug treatment failure were previously identified drug resistance mutations in subtype B infection. Substitutions associated with drug resistance to azidothymidine (AZT), lamivudine (3TC), and didanosine (ddI) in subtype B infections were also frequently found in CRF01_AE drug-resistant patients as previously reported.[16] A two-amino acid insertion at position 67, which we have previously demonstrated as conferring multiple RT inhibitor resistance in subtype B infection, was also seen in CRF01_AE viruses.[17] Our data showed that the two mutations, T69N and V75M, in the RT differ in frequency, however. It is worth noting that the V75M mutation was more commonly seen in CRF01_AE infection and significantly associated with d4T therapy in CRF01_AE infection but not in subtype B infection. The difference was marginal, and further investigation will be required to confirm the clinical significance of this finding. Furthermore, we have shown that several drug resistance mutations in the protease region appear to be characteristic of CRF01_AE infection. In particular, the known mutations at L10F, K20I, L33I, and N88S were found more frequently in CRF01_AE infection than in subtype B infection. Our data suggest that N88S is an important drug resistance mutation presumably against nelfinavir in CRF01_AE infection. The N88S has been reported to confer drug resistance against nelfinavir, indinavir, and BMS-232632 in subtype B[18]; however, due to the rarity of subtype B clinical samples, further studies are still required to clarify the implication of the N88S mutation.[19]

Interestingly, the N88S mutation was strongly linked with the L10F mutation in CRF01_AE patients ($p < .0001$); all 6 CRF01_AE patients with the N88S mutation also had the L10F mutation. Conversely, there were 3 subtype B patients with L10F and 4 subtype B patients with N88S, none of who had both mutations. Surprisingly, none of the PI-resistant CRF01_AE patients had D30N, A71V, or N88D, which are often found in PI-resistant subtype B patients. As previously reported, our observation also showed a strong link between D30N and N88D in subtype B patients ($p < .0001$); 21 of 22 subtype B patients with the N88D mutation also had the D30N mutation.[20]

A number of studies have shown the benefit of having genotype results for determining the optimal drug regimen in subtype B infections.[21,22] Our data suggest that the knowledge acquired from subtype B drug resistance genotypes cannot always be applied to interpret subtype CRF01_AE genotype results, however, especially when interpreting genotypic results from patients receiving nelfinavir. Accumulation of data and analysis of drug resistance mutations in non-B subtype infections are urgently needed to improve the selection of the optimum drug regimen specific for each subtype.

Our results were derived from a cross-sectional and retrospective observation with rather small data sets and do not exclude the possibility that these drug-resistance mutations existed as naturally occurring polymorphisms prior to antiretroviral treatment. Nevertheless, there were no such polymorphisms in the drug-naive patients in our data and previous reports of the *pol* gene analysis of non-B subtypes,[11] suggesting that this case is highly unlikely. Another limitation is that in the current study, we did not have information about drug adherence, which may have caused a significant proportion of treatment

failure. Further studies using prospective cohort patients with drug adherence data will be essential to confirm these preliminary findings. With much larger data sets, we may be able to tease out more minor differences. We found that these patients with CRF01_AE infection had significantly more advanced HIV disease than the subtype B patients and that the sex ratio was significantly different. We believe that these differences do not account for the difference in the patterns of drug resistance mutation, however. We were also concerned about a potential bias induced by a group of patients for whom protease sequence data were not available. The proportion of female patients in this group was 20% and that of heterosexual transmission was 49%. These proportions were higher than those for subtype B infection and lower than those for CRF01_AE infection. We suspected that this group is a mixture of both subtype B and CRF01_AE patients who were successfully treated; thus, it should not affect the results among treatment failure groups.

In summary, we have observed some unique patterns of mutations in the *pol* gene of CRF01_AE-infected patients who failed to respond to antiretroviral drug treatment. Our data strongly suggest that CRF01_AE viruses evolve differently from subtype B viruses under the selection pressure of combination antiretroviral therapy, particularly in relation to nelfinavir. It is now important to expand our knowledge of drug-resistant genotypes in widely prevailing non-B subtype HIV viruses.

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Brief Report

Survival Benefit from Non–Highly Active Antiretroviral Therapy in a Resource-Constrained Setting

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Summary: Mortality rates among HIV-1–infected patients attending a government hospital in northern Thailand were investigated to evaluate the effect of antiretroviral (ARV) drug therapy on mortality. Demographic, clinical, and laboratory data and history of ARV drug therapy were collected from all HIV-1–infected adult patients who attended the Day Care Center clinic from October 2, 1995 through October 31, 1999. The survival status of patients until October 31, 1999 was ascertained from the hospital records, mailing letters, and death certificates at the Provincial Health Office. Of 1110 patients who attended the clinic, we had data on duration of follow-up for 1081 (97%) with a total of 1175 person-years of observation; 607 (54.7%) patients died. Clinical status, CD4 group, ARV drug group, and registered year were independently associated with death. The adjusted hazard ratio of monotherapy to no therapy was 0.65 (95% CI: 0.48, 0.87; $p = .001$) and that of dual therapy was 0.43 (95% CI: 0.29, 0.62; $p < .001$). The mortality rate of patients attending a government hospital in northern Thailand is high. Suboptimum ARV drug regimens like dual therapy had a substantial survival benefit. Further cost reduction for multiple ARV drug regimens is impatiently awaited. **Key Words:** HIV—Antiretroviral drug—Dual therapy—Mortality—Thailand.

Thailand is known to be the first and probably the most severely affected area of the HIV-1 epidemic in Southeast Asia (1). Studies on the mortality rate of HIV-1–infected patients remain limited in the region, however (2,3). The clinical setting, availability of treatment (especially antiretroviral [ARV] drugs), spectrum of opportunistic diseases, and genetic backgrounds are all thought to affect the mortality rate. More information on mortality rates of HIV-1–infected patients is thus necessary, because it is useful to estimate disease burden on the community and local hospitals.

There is ample evidence indicating that triple ARV drug therapy can improve the quality and length of an HIV-1–infected patient's life (4). Triple therapy is not always available to patients living in developing countries, however. In Thailand, it was not until recently that the government had a policy to provide triple therapy to HIV-1–infected individuals on a select basis. Before then, HIV-1–infected patients were either not treated or

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