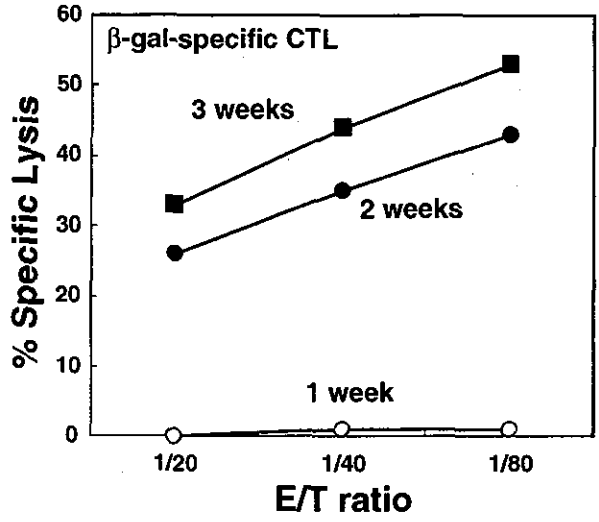

Fig. 5 Kinetics of β-gal-specific CTL activity.
Two weeks (●) and 3 weeks (■) after gene gun immunization with gold beads coated with β-gal plasmid, strong CTL activity against BALB/c 3T3 fibroblast targets pre-sensitized with the known 9-mer CTL epitope peptide (TPHPARIGL) was observed, though one week (○) after the immunization no specific cytotoxicities were detected. Results are representative of five independent experiments.

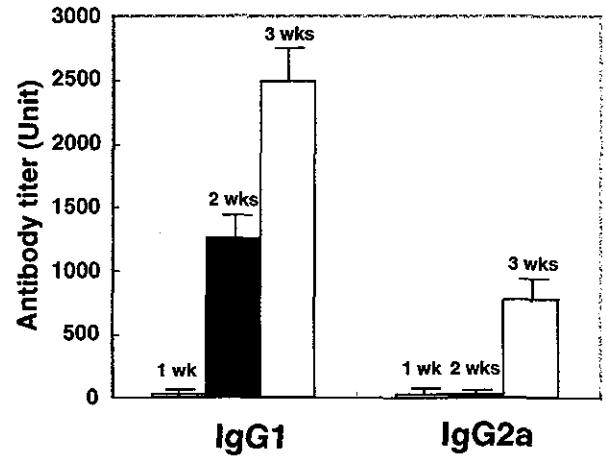

Fig. 6 Kinetics of β-gal-specific IgG1 and IgG2a antibody production.
Two weeks after gene gun immunization, a β-gal-specific Th2-type of IgG1 antibody response was observed, whereas a β-gal-specific Th1-type of IgG2a antibody response could be detected at least three weeks after the immunization. Results are representative of three independent experiments and shown as the mean ± SD of four animals in each group.

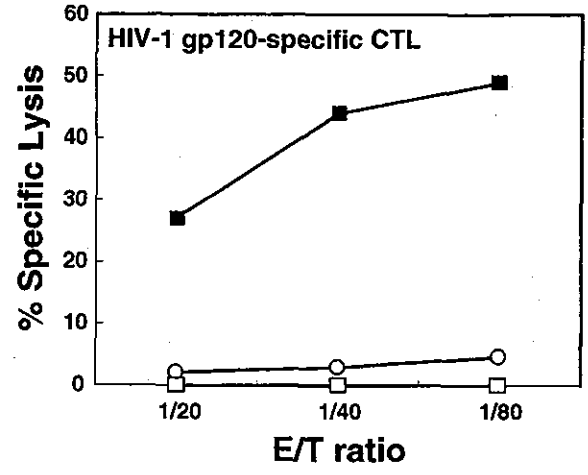

Fig. 7 Induction of HIV-1 gp120-specific CTL with DNA plasmids.
BALB/c mice were immunized with three shots of gold beads coated with HIV-1 gp120 DNA plasmid together with an equal amount of β-gal plasmid using gene gun. Three weeks after the immunization, gp120-specific CTL could be generated from immune spleen cells (■). However, when the mice were imuunized with a mixture of two distinct gold beads coated either with β-gal or gp120 plasmid (○), or immunized with gold beads coated gp120 plasmid alone (□), could not prime HIV-1-specific CTL. Results are representative of three independent experiments.

same antigen (14). In contrast, Robinson *et al.* found that direct injection of influenza hemaggulutinin (H1)-expressing plasmid in saline raised a predominantly Th1 response with mostly IgG2a antibody production, while gene gun immunization with the same plasmid predominantly produced a Th2 response with mostly H1-specific IgG1 antibody production (5). Our results seem to reflect a combination of those observations in that β-gal-specific $CD8^+$ CTL reflecting a Th1 type of response were primed, while a Th 2 type of IgG1 antibody to β-gal was dominantly produced 2 weeks after the gene gun immunization with β-gal plasmid. This discrepancy of Th1 and Th2 balance in the β-gal-specific responses might be because β-gal-specific CTL were independently primed by activated DC without requiring a Th1 type of help as demonstrated recently (15, 19).

After confirming the difficulty in induction of CTL specific for HIV-1 gp120 by immunization with multiple gene gun bombardments of the gold beads coated with HIV-1 gp120 plasmid DNA having CMV promoter but not ISS sequence, we found that the gp120-specific $CD8^+$ CTL could be efficiently primed when mice were immunized with the gold beads coated with less than 1 μg of gp120 plasmid DNA together with equal amount of β-gal plasmid. It is of note that the gp120-specific $CD8^+$ CTL could not be primed when mice were immu-

nized with a mixture of two distinct gold beads coated either with β-gal or gp120 plasmid. The finding suggests that the gp120 plasmid should be captured by the intradermal immature DC together with the β-gal plasmid when the amount of loaded plasmid was very small. Also, the DC that caught the gold beads bearing gp120 plasmid are hard to be activated by the external plasmid having CpG motif presented by another gold beads. Thus, the antigen-captured dermal DC may be activated by the internal β-gal plasmid via CpG DNA and toll-like receptor 9 interaction (10). Taken together, gp120-specific CTL might be primed by the activated DC which captured gold beads bearing both β-gal and gp120 plasmids.

These findings of the present study should prove useful for the development of DNA vaccines that can be used to control a variety of diseases, such as infectious diseases, cancers, and autoimmune disorders, by activating $CD8^+$ CTL with a small amount of plasmid inoculated intradermally by gene gun.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Science, Sport, and Culture, from the Ministry of Health and Labor and Welfare, Japan, and from the Japanese Health Sciences Foundation, and by the Promotion and Mutual Aid Corporation for Private School of Japan.

REFERENCES

1. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A and Martin MA (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59**, 284–291.
2. Akahata W, Ido E, Akiyama H, Uesaka H, Enose Y, Horiuchi R, Kuwata T, Goto T, Takahashi H and Hayami M (2003) DNA vaccination of macaques by a full-genome simian/human immunodeficiency virus type 1 plasmid chimera that produces non-infectious virus particles. *J Gen Virol* **84**, 2237–2244.
3. Akahata W, Ido E, Shimada T, Katsuyama K, Yamamoto H, Uesaka H, Ui M, Kuwata T, Takahashi H and Hayami M (2000) DNA vaccination of macaques by a full genome HIV-1 plasmid which produces noninfectious virus particles. *Virology* **275**, 116–124.
4. Chapman BS, Thayer RM, Vincent KA and Haigwood NL (1991) Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res* **19**, 3979–3986.
5. Feltquate DM, Heaney S, Webster RG and Robinson HL (1997) Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* **158**, 2278–2284.
6. Fujimoto C, Nakagawa Y, Ohara K and Takahashi H (2004) Polyriboinosinic polyribocytidylic acid [poly(I:C)]/TLR3 signaling allows class I processing of exogenous protein and induction of HIV-specific $CD8^+$ cytotoxic T lymphocytes. *Int Immunol* **16**, 55–63.
7. Fuller DH and Haynes JR (1994) A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA- based glycoprotein 120 vaccine. *AIDS Res Hum Retroviruses* **10**, 1433–1441.
8. Gavin MA, Gilbert MJ, Riddell SR, Greenberg PD and Bevan MJ (1993) Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. *J Immunol* **151**, 3971–3980.
9. Gurunathan S, Klinman DM and Seder RA (2000) DNA vaccines: immunology, application, and optimization. *Annu Rev Immunol* **18**, 927–974.
10. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K and Akira S (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
11. Nichols WW, Ledwith BJ, Manam SV and Troilo PJ (1995) Potential DNA vaccine integration into host cell genome. *Ann N Y Acad Sci* **772**, 30–39.
12. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF and McMichael AJ (1998) Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**, 2103–2106.
13. Pantaleo G, Demarest JF, Soudeyns H, Graziosi C, Denis F, Adelsberger JW, Borrow P, Saag MS, Shaw GM, Sekaly RP and Fauci AS (1994) Major expansion of $CD8^+$ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* **370**, 463–467.
14. Raz E, Tighe H, Sato Y, Corr M, Dudler JA, Roman M, Swain SL, Spiegelberg HL and Carson DA (1996) Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* **93**, 5141–5145.
15. Ridge JP, Di Rosa F and Matzinger P (1998) A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature* **393**, 474–478.
16. Rouse RJ, Nair SK, Lydy SL, Bowen JC and Rouse BT (1994) Induction in vitro of primary cytotoxic T-lymphocyte responses with DNA encoding herpes simplex virus proteins. *J Virol* **68**, 5685–5689.
17. Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA and Raz E (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
18. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scallon BJ, Ghrayeb J, Forman MA, Montefiori DC, Rieber EP, Letvin NL and Reimann KA (1999) Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. *Science* **283**, 857–860.
19. Schoenberger SP, Toes RE, van der Voort EI, Offringa R and Melief CJ (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480–483.
20. Sindhu ST, Ahmad R, Blagdon M, Ahmad A, Toma E, Morisset R and Menezes J (2003) Virus load correlates inversely with the expression of cytotoxic T lymphocyte activation

markers in HIV-1-infected/AIDS patients showing MHC-unrestricted CTL-mediated lysis. *Clin Exp Immunol* **132**, 120–127.

21. Takahashi H, Cohen J, Hosmalin A, Cease KB, Houghten R, Cornette JL, DeLisi C, Moss B, Germain RN and Berzofsky JA (1988) An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc Natl Acad Sci USA* **85**, 3105–3109.
22. Takahashi H, Nakagawa Y, Leggatt GR, Ishida Y, Saito T, Yokomuro K and Berzofsky JA (1996) Inactivation of human immunodeficiency virus (HIV)-1 envelope-specific $CD8^+$ cytotoxic T lymphocytes by free antigenic peptide: a self-veto mechanism? *J Exp Med* **183**, 879–889.
23. Takahashi H, Nakagawa Y, Yokomuro K and Berzofsky JA (1993) Induction of $CD8^+$ cytotoxic T lymphocytes by immunization with syngeneic irradiated HIV-1 envelope derived peptide-pulsed dendritic cells. *Int Immunol* **5**, 849–857.
24. Takahashi M, Osono E, Nakagawa Y, Wang J, Berzofsky JA, Margulies DH and Takahashi H (2002) Rapid induction of apoptosis in $CD8^+$ HIV-1 envelope-specific murine CTLs by short exposure to antigenic peptide. *J Immunol* **169**, 6588–6593.
25. Vabulas RM, Pircher H, Lipford GB, Hacker H and Wagner H (2000) CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J Immunol* **164**, 2372–2378.
26. Warren TL, Bhatia SK, Acosta AM, Dahle CE, Ratliff TL, Krieg AM and Weiner GJ (2000) APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J Immunol* **165**, 6244–6251.
27. Yasutomi Y, Robinson HL, Lu S, Mustafa F, Lekutis C, Arthos J, Mullins JI, Voss G, Manson K, Wyand M and Letvin NL (1996) Simian immunodeficiency virus-specific cytotoxic T-lymphocyte induction through DNA vaccination of rhesus monkeys. *J Virol* **70**, 678–681.

Study of Antiretroviral Drug–Resistant HIV-1 Genotypes in Northern Thailand: Role of Mutagenically Separated Polymerase Chain Reaction as a Tool for Monitoring Zidovudine-Resistant HIV-1 in Resource-Limited Settings

Siriphan Saeng-Aroon, MSc, Nuanjun Wichukchinda, MSc,* Lay Myint, MD, PhD,† Panita Pathipvanich, MD,‡ Koya Ariyoshi, MD, PhD,*† Archawin Rojanawiwat, MD,* Masakazu Matsuda,† Pathom Sawanpanyalert, MD, PhD,* Wataru Sugiura, MD, PhD,† and Wattana Auwanit, PhD**

Summary: As the number of HIV-1–infected individuals receiving antiretroviral drugs has been rapidly increasing in developing countries, there is an urgent need for drug resistance genotype information of non-B subtype HIV-1 and for the establishment of a practical system of monitoring drug-resistant viruses. This study first sequenced the reverse transcriptase region of HIV-1 in 112 infected individuals who had been treated with zidovudine (AZT)/didanosine or AZT/zalcitabine as dual therapy at a government hospital in northern Thailand and then compared the above sequence method with mutagenically separated polymerase chain reaction (MS-PCR) for detecting M41L and K70R mutations. Concordant rates of detecting M41L and K70R mutations by the 2 methods were 96.9% (93/96) and 92.7% (89/96), respectively. The M41L and K70R MS-PCR could detect 86.4% of AZT-resistant strains with any resistance mutation, which was determined by the sequencing method. Then 292 drug-naive individuals were screened for the presence of drug-resistant HIV-1 by the MS-PCR assay and it was found that 2 individuals (0.7%) carried viruses with either the M41L or K70R mutation. It is feasible to test a large number of samples with MS-PCR, which is sensitive, cheap, and easy to perform and does not require sophisticated equipment. The M41L and K70R MS-PCR is potentially a useful tool to monitor the spread of AZT-resistant HIV-1 in resource-limited countries.

Received December 1, 2003; accepted for publication May 7, 2004.

From *National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; †AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; and ‡Day Care Center, Lampang Hospital, Lampang, Thailand.

Supported by the Department of Medical Sciences, Ministry of Public Health of Thailand, under the national plan for AIDS prevention and control, the Japan International Cooperation Agency (JICA), and the Ministry of Health, Social Welfare and Labour of Japan.

Reprints: Wattana Auwanit, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand 11000 (e-mail: wattana@dmsc.moph.go.th).

Key Words: HIV, CRF01_AE, Thailand, antiretroviral drug, drug resistance

(*J Acquir Immune Defic Syndr* 2004;36:1051–1056)

HIV-1 has tremendous ability to mutate swiftly and to develop resistance to almost all clinically used antiretroviral drugs. Reduced sensitivity to nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTI (NNRTIs), and protease inhibitors has been studied extensively and is linked to specific point mutations in either the reverse transcriptase or the protease gene.[1] Most current knowledge for interpreting these genotypic changes has been derived from studies on HIV-1 subtype B viruses. Worldwide, however, the majority of HIV-1–infected people live in developing countries and most of them are infected with non-B subtypes. Non-B subtypes differ from subtype B in *pol* gene by 10–15%.[2] We and other groups have published data showing some discrete differences in the patterns of drug resistance mutations between subtypes.[3–5] With a growing demand for access to antiretroviral therapy in resource-limited countries, the resistance patterns of non-B subtype viruses to antiretroviral drugs are becoming an important issue.

In Thailand, with a population of approximately 62 million, it was estimated that 695,000 people were living with HIV-1 infection. Of these, the majority are infected with CRF01_AE (previously known as subtype E), and 55,000 people had AIDS in 2000.[6] In the past, only a small minority of HIV-1–infected patients could afford antiretroviral drugs due to the high monthly price; thus most were either not treated or were treated with suboptimal antiretroviral regimens, mostly dual therapy.[7] When patients are treated suboptimally, HIV-1 acquires resistance to drugs more quickly.

Furthermore, the prevention of mother-to-child HIV-1 transmission (PMTCT) program, which uses a short-course zidovudine (AZT) regimen, was expanded first in northern Thailand in 1997 and later throughout the country.[8] This expanded program could also trigger the emergence of AZT drug resistance. Studies of HIV-1–infected individuals with primary HIV infection have shown that drug-resistant HIV strains can be transmitted from one adult to another and occasionally transmitted vertically from mother to child.[9–12] To control the spread of drug-resistant HIV-1, a monitoring system of antiretroviral drug-resistant HIV-1 in an epidemiologic scale is urgently needed.

The *pol* gene has been commonly sequenced for testing drug resistance in many HIV laboratories of developed countries. However, access to the sequencing test in developing countries is limited due to the relatively high costs of reagents and unavailability of expensive equipment such as an automated sequencer. Polymerase chain reaction (PCR)-based assays are an alternative method of detecting point mutations, having the advantage of increased sensitivity and low cost. Allele-specific primer extension assays have been applied to detect drug-resistant HIV-1; however, they have not been adequately specific for widespread application.[13,14] Conversely, mutagenically separated PCR (MS-PCR) is a PCR-based point mutation assay that overcomes this specificity limitation and has been successfully applied to detect drug-resistant HIV-1 of non-B subtypes.[15–17] Previous papers evaluated the performance of MS-PCR in developed countries, but it has not yet been used in resource-limited settings.

We conducted this study with the following objectives: to evaluate the performance of MS-PCR specific for M41L and K70R mutations in detecting AZT-resistant HIV-1 strains in Thailand; to apply the MS-PCR to the screening of AZT-resistant HIV-1 among drug-naive HIV-1–infected Thais; and to investigate the patterns and prevalence of drug-resistant genotypes among HIV-1–infected Thai individuals who had been treated with suboptimal antiretroviral regimens by sequencing the *pol* gene.

PATIENTS AND METHODS

Study Population

We used samples obtained from HIV-1–infected individuals who attended the Day Care Center clinic at the Lampang Hospital from July 6, 2000, to July 15, 2001 and gave a written informed consent. The Lampang HIV study was approved by the Thai government ethics committee. The Lampang Hospital is a government referral hospital with approximately 800 beds, situated in the center of Lampang province, which is 100 km south of Chiang Mai in northern Thailand. Plasma samples were collected from these individuals and stored at −80°C until their use. Viral load measurement was conducted using a commercial kit (Amplicor HIV-1 Monitor Test, version 1.5; Roche Diagnostics, Branchburg, NJ). $CD4^+$ cell count was measured by flow cytometory (FACScan; Becton-Dickinson, Franklin Lakes, NJ).

Direct Sequencing

After viral load measurement, the residual RNA was used for drug resistance genotyping by sequencing *pol* gene as previously described.[17] Briefly, an 888-basepair (bp) reverse transcriptase fragment (base number of nucleotide: 2485–3372) was amplified by PCR after a reverse transcription (RT) reaction from the RNA by an RNA-PCR kit (AMV One Step RNA PCR Kit; Takara, Osaka, Japan). Primary PCR products were further amplified with a high-fidelity DNA polymerase (KOD DNA polymerase; Toyobo, Osaka, Japan). Sequencing was performed using an autosequencer ABI-3100 (Applied Biosystems, Foster City, CA) with dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems). The sequence results were assembled and aligned on the reference HIV sequence of HIV-1 HXB2 (GenBank accession number M38432) by ABI Prism SeqScape Software (Applied Biosystems). They were submitted to Stanford HIV RT and Protease Sequence Database (http://hivdb.stanford.edu/) for drug resistance genotyping.

M41L and K70R MS-PCR

On measuring the viral load, we used the residual RNA for the MS-PCR experiments. The method of MS-PCR for detecting M41L and K70R AZT resistance mutations in CRF01_AE has been described in a previously published paper.[17] Briefly, the first-round RT-PCR was conducted to amplify a 370-bp RT region, which spans codon 41 and 70. The second- and third-round PCR were conducted with 1 common forward primer and 2 reverse primers, which are allele specific. The size of the wild-type specific primer was designed about 20 nucleotide bases longer than the mutant type-specific primer so that the wild-type PCR product could be easily differentiated from the mutant-type PCR product by electrophoresis with a 3% agarose gel. We used clinical samples, of which drug-resistant genotypes were confirmed by the sequencing method used for positive controls, and included them for every experiment. When an MS-PCR result showed double bands, of which one was faint, we retested the sample.

Statistical Methods

We compared proportions by the χ^2 test. Means of continuous variables were compared by a nonparametric test, the Kruskal-Wallis one-way analysis of variance. The data management and statistical analysis were conducted using Epi Info version 6.04.

RESULTS

A total of 489 HIV-1–infected individuals attended the clinic during the observation period. History of antiretroviral

drug therapy was available from 487 infected individuals, in whom 336 were drug naive and 151 were drug experienced; 22 individuals were exposed to a single NRTI, 114 to two NRTIs, 14 to three or more antiretroviral drugs including protease inhibitors, and one did not have regimen information.

Patterns and Prevalence of Drug Resistance Mutations Among Suboptimally Treated Individuals

Out of the 114 individuals who had been exposed to 2 NRTIs, we first analyzed 112 individuals who had received AZT/didanosine (ddI) or AZT/zalcitabine (ddC) dual therapy for the sequencing of RT region; the other 2 individuals had received either d4T/ddC or AZT/lamivudine (3TC). A total of 108 individuals (96.4%) were infected via the heterosexual route. The median (range) duration of dual therapy was 621 (28–1600) days; 76 individuals were on therapy and 36 patients were off therapy at the time of sampling; 28 individuals were exposed to AZT/ddI, 76 to AZT/ddC, and 8 to both AZT/ddI and AZT/ddC. Sequence data were obtained from 99 individuals, of whom 57 (57.6%) had mutations that are known to be associated with AZT, ddI, or ddC resistance. We could not obtain sequence data from 13 individuals, mainly because of their low viral load; 10 had a viral load under the detectable level (<400 copies/mL). Assuming that the individuals without sequence data did not have any drug-resistant virus, the prevalence of drug-resistant viruses detected by the sequencing method was 57/112 (50.9%) among those who had received dual therapy. If confined to the 76 individuals on therapy, 48 (63.2%) had drug-resistant viruses. Figure 1 summarizes amino acid variations at the sites, which are known to be associated with AZT, ddI, or ddC resistance mutations in subtype B infection. The most common drug resistance mutations were D67N, followed by K70R and T215Y/F. Other mutations were also commonly seen at codon 41, 210, and 219. No mutation of Q151M was found. We also analyzed associations among these specific mutations. The presence of D67N was strongly associated with K70R and less significantly with M41L; 31 (86.1%) out of 36 individuals with D67N mutation had either a K70R or M41L mutation (Table 1). The presence of T215Y/F was strongly associated with M41L but not with K70R; 22 (71%) of 31 individuals with T215Y/F mutation had K70R or M41L mutation (Table 1).

TABLE 1. Associations of D67N and T215Y/F With M41L and K70R Mutations

Codon 67 Mutation						
	M41L*		K70R†		M41L or K70R*	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Codon 67						
D	57	6	55	8	49	14
N	26	10	12	24	5	31

*$P < 0.0001$, †$P = 0.017$.

Codon 215 Mutation						
	M41L*		K70R		M41L or K70R†	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Codon 215						
T	66	2	46	22	45	23
Y/F	17	14	21	10	9	22

*$P < 0.0001$, †$P = 0.0005$.

Drug Resistance Mutations in Relation to the Duration of Antiretroviral Therapy

The prevalence of drug resistance mutations correlated with the duration of dual therapy among 76 individuals on therapy. Among those with the duration of therapy for <180 days, 180–365 days, and >365 days, drug-resistant viruses were found to be predominant in 3 (30%), 8 (62%), and 33 (69%) individuals, respectively, with the median numbers of drug resistance mutations of 0, 2, and 2, respectively (Fig. 2). The number of drug resistance mutations was significantly associated with the median level of viral load: the median (interquartile range, IQR) viral load of individuals with no mutation, with 1–4 mutations, and with ≧5 mutations was 7412 (<400–62,432); 37,871 (7866–105,105); and 156,989 (32,682–184,767) copies/mL, respectively ($P = 0.018$ by Kruskal Wallis one-way analysis of variance).

Codon	41	44	65	67	69	70	74	108	118	151	184	210	215	219
Consensus B	M	E	K	D	T	K	I	V	V	Q	M	L	T	K
N=99	L(12) V(1) I(2)	D(3)	V(1)	N(33) E(2) G(2)	D(1) N(2) S(1) S_SG(1)	R(29)	V(1)	-	I(6)	-	-	W(13) F(2) M(1)	Y/ F(28) I(10) S(2) N(1)	Q/ E(20) N(1)

FIGURE 1. Patterns of AZT, ddI, or ddC resistance mutations. This figure shows amino acid variation at known AZT, ddI, or ddC resistance mutation sites. The frequency of each substitution is shown in parentheses. Reported drug resistance–associated mutations are shown in bold.

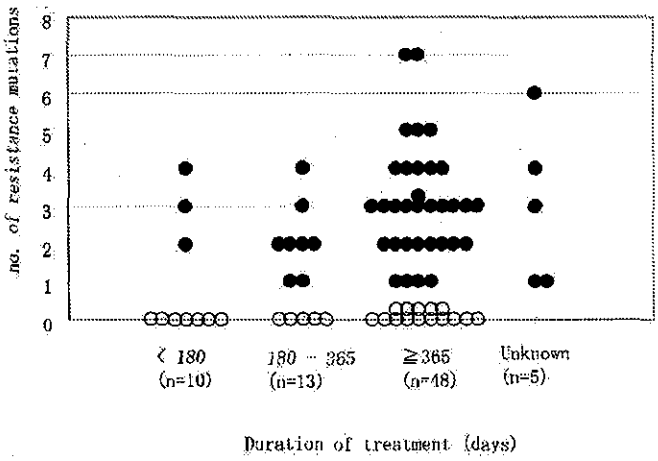

FIGURE 2. Prevalence of AZT, ddI, or ddC resistance mutations in relation to the duration of the dual therapy. Open circles indicate samples without resistance mutations.

Drug Resistance Mutations After Stopping Antiretroviral Drug Therapy

The interval between stopping dual therapy and the time of sampling was also associated with the presence of drug resistance mutations. Twenty-six individuals who had been substantially exposed to dual therapy for >180 days were off therapy at the time of sampling. Seven individuals (27%) were off therapy for >180 days and 19 (73%) were off therapy for <180 days. Drug-resistant viruses were detected in only 1 individual (14%) in the former group but 7 individuals (37%) in the latter group.

Evaluation of M41L and K70R MS-PCR in Detecting AZT-Resistant Strains

We then compared direct sequence methods with M41L and K70R MS-PCR in 96 antiretroviral drug–experienced individuals for whom both sequence and MS-PCR results were available (Table 2). Overall concordant rate for codon 41 was 96.9% (93/96) where M41I was regarded as a mutant type and concordant rate for codon 70 was 92.7% (89/96). Discordant results were seen mainly in the samples that were determined as mutant type by the MS-PCR and as wild type by the sequencing method.

To study the sensitivity of M41L and K70R MS-PCR as a screening strategy in detecting AZT-resistant strains, we defined the AZT-resistant strains as viruses with at least one AZT resistance mutation, which was detected by the sequencing method. Out of the 96 plasma samples that were tested by both the sequencing and the MS-PCR methods, 52 samples had no AZT resistance mutation and 44 samples had at least one AZT resistance mutation and were regarded as containing AZT-resistant viruses. Of the 44 samples with AZT-resistant viruses, the M41L and K70R MS-PCR detected either M41L or K70R mutation in 38 samples, resulting in the sensitivity of the M41L and K70R MS-PCR in detecting the AZT-resistant viruses at 86.4%. The number of AZT resistance mutations related to the detection rate of AZT resistance mutations by the MS-PCR (Table 3). When the viruses had multiple mutations, the sensitivity of the M41L and K70R MS-PCR was considerably higher. Of 39 samples containing HIV-1 with more than one AZT resistance mutation, 37 samples (94.5%) were diagnosed as having resistant viruses by the M41L and K70R MS-PCR.

TABLE 2. Comparison Between MS-PCR and Sequencing Results

Codon 41 Mutations			
	Sequencing Results		
	M	L	I
MS-PCR results			
Wild	84	1*	0
Mutant	2	7	2

*Sequence result of this patient showed a mixed type of M and L; it turned out to be mutant type when MS-PCR experiment was repeated.

Codon 70 Mutations		
	Sequencing Results	
	K	R
MS-PCR results		
Wild	65	1
Mutant	6	24

Screening AZT-Resistant Viruses Among Antiretroviral Drug–Naive Individuals in Northern Thailand

We applied the M41L and K70R MS-PCR to the screening of 292 antiretroviral drug–naive HIV-1–infected individu-

TABLE 3. The Sensitivity of M41L and K70R MS-PCR in Detecting AZT Resistance Mutations

AZT Resistance Mutations, n	Total	M41L and K70R MS-PCR Results		
		Wild	Mutant	Sensitivity
0	52	52	0	—
1	5	4	1	20%
2	14	1	13	92.9%
3	11	1	10	90.9%
≥4	14	0	14	100%

als attending the Lampang Hospital for the existence of AZT drug-resistant viruses. There were 271 individuals (92.8%) who were known to be infected with HIV-1 via the heterosexual route. We found 2 patients (0.7%) who carried mutant viruses: one had M41L and the other had K70R mutation. Later it was noted that these 2 patients, as well as their spouses, had never received any antiretroviral drugs but both had participated in clinical trials of herbal medicine in the past.

DISCUSSION

Our observation showed that AZT, ddI, or ddC resistance mutations were found in >50% of individuals who had received dual therapy. The prevalence of drug-resistant viruses was higher among individuals who had received the drugs for a longer period, as previously reported.[18,19] We attribute the high prevalence of resistant viruses to the fact that the dual therapy was suboptimal. Clinicians working in government hospitals, however, did not have other options because the more efficient antiretroviral therapy such as triple or quadruple therapy was not affordable for most patients when this study was conducted.[7] Recently, access to multiple antiretroviral drugs has been dramatically improved, because the Government Pharmaceutical Organization (GPO) started the production of generic antiretroviral drugs known as "GPOvir," which is a combined tablet of stavudine, lamivudine, and nevirapine. We nevertheless anticipate that individuals who had already had viruses resistant to NRTI dual therapy may not gain as much benefit from the generic medicine as antiretroviral drug–naive individuals do.

The most common mutations observed in this study were D67N, K70R, and T215Y/F, and we found few mutations at codons 65, 74, 108, 151, and 184. Such patterns of NRTI resistance mutations are similar to the patterns in CRF01_AE infection as well as in subtype B infections that have been reported in our previous report.[4] M184V mutation was often found in our previous study but not in the current study. We think that this difference reflects on the rare use of 3TC in Thailand when this study was conducted. Our current study, though a cross-sectional observation, showed several associations among resistance mutations such as D67N and M41L or K70R, T215Y/F and M41L in Thai strains as known in subtype B infection.[20,21]

We found a high concordance rate of MS-PCR with the sequencing method in detecting M41L and K70R point mutations. The finding is compatible with previous papers.[16,17] Discordant results between the MS-PCR and sequencing method were seen in some samples, most of which showed mutant type by the MS-PCR but wild type by the sequencing method. We think that such discordances are due to the greater sensitivity of MS-PCR for detecting a minor virus population than the sequencing method. However, a high sensitivity and specificity of detecting 2 particular point mutations do not specifically justify the application of M41L and K70R MS-PCR for the screening of AZT-resistant viruses in the field. D67N and T215Y/F mutations are very common but it is technically difficult to establish MS-PCR specific for these mutations due to a higher degree of polymorphism around the mutation sites. Our data showed that these mutations were frequently accompanied by M41L and/or K70R as previously reported in subtype B.[22] Furthermore, we evaluated how efficiently the M41L and K70R MS-PCR could detect AZT-resistant viruses that were detected by the sequencing. The overall sensitivity was reasonably high particularly among the viruses with multiple drug resistance mutations.

This is the first report that addressed the transmission of drug-resistant HIV-1 using a large number of samples in Thailand. We found that the prevalence of HIV-1 strains with either M41L or K70R mutation was as low as 0.7% among our drug-naive population. Considering that the overall sensitivity of the MS-PCR for detecting HIV-1 with any AZT resistance mutation was 86.4%, the prevalence of AZT-resistant HIV-1 was estimated to be 0.8%, which is still very low. There is still the concern that the low prevalence of resistant virus could be a consequence of the fact that the resistance to AZT in the drug-naive population was often associated with mutations at codon 60 or 215. To exclude this possibility, we further tested 60 samples, which were randomly selected from the drug-naive samples and confirmed that none had drug resistance mutations at these sites. The majority (127/292) of drug-naive individuals (43.5%) were initially diagnosed as HIV infected in 1997 or before, when the PMTCT program started in the region, and many were likely to have been infected several years prior to their first diagnosis of HIV infection. Thus, our result may not show an effect, which could have been triggered by the PMTCT program. A report from the United Kingdom suggests that transmission of drug-resistant HIV-1 is increasing.[23] We believe that our report is important in providing the baseline information on AZT-resistant HIV-1.

There has not been a consensus on the strategy of monitoring the transmission of drug-resistant HIV-1 in developing countries. Detecting individuals with primary viremia is ideal but not practical. In our study, we surveyed a drug-naive population for the presence of drug-resistant viruses. One concern with this approach is that drug-resistant viruses, which are generally less fit, might have been overwhelmed by the wild-type viruses in the absence of antiretroviral drug pressure because drug-resistant viruses among drug-treated individuals disappear following the interruption of antiretroviral therapy.[24] However, a recently published paper showed 2 cases of transmission of drug-resistant HIV-1 in which the resistant genotypes remained as a dominant population for a prolonged period in the absence of antiretroviral therapy.[25] Another way to monitor the spread of antiretroviral drug–resistant viruses is to screen infected individuals shortly after they receive antiretroviral therapy, which selects a minor population of insidious

resistant viruses, before de novo resistance mutations occur. Further studies are needed.

This study demonstrates that it is feasible to apply MS-PCR techniques for screening a large number of field samples for the presence of AZT-resistant viruses in Thailand. Taking into account the enormous benefits of MS-PCR such as much lower cost, ease of use, no requirement of automated sequencers, and higher sensitivity of detecting a minor virus population, we think that the M41L and K70R MS-PCR is a useful technique for the screening of AZT-resistant HIV-1 in epidemiologic surveys in developing countries. Recently, GPOvir has become widely available in Thailand. As the patterns of drug-resistant mutations against 3TC and nevirapine are relatively simple, we propose that MS-PCR technique should be considered for monitoring viruses resistant to this combination of antiretroviral drugs.

ACKNOWLEDGMENTS

We thank Ms. Suthira Kasemusuk, Ms. Sriprai Seneewong-na-ayoottaya, Ms. Anong-nard Ariyakrua, Ms. Nutira Boonna, and Mr. Praphan Wrongramnong for their field works; Ms. Kanha Lor-yont, Mr. Wicharn Khaewkarnka, Mr. Sithichai Umnajsirisuk, Mr. Somchai Ni-yom-thai, and Ms. Jirawan Srisawat for their laboratory assistance. Finally, we would like to thank the HIV-infected individuals who participated in this study.

REFERENCES

1. Hirsch MS, Brun-Vezinet F, Clotet B, et al. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an international AIDS society-USA panel. *Clin Infect Dis*. 2003;37:113–128.
2. Robertson DL, Anderson JP, Bradac JA, et al. HIV-1 nomenclature proposal, 1999. Available at: http://hiv-web.lanl.gov/conent/hiv-db/REVIEWS/review.html. Accessed on October 31, 2000.
3. Grossman Z, Vardinon N, Chemtob D, et al. Genotypic variation of HIV-1 reverse transcriptase and protease: comparative analysis of clade C and clade B. *AIDS*. 2001;15:1453–1460.
4. Ariyoshi K, Matsuda M, Miura H, et al. Patterns of point mutations associated with anti-retroviral drug resistance in CRF01_AE (subtype E) infection differ from subtype B infection. *J Acquir Immune Defic Syndr*. 2003;33:336–342.
5. Brenner B, Turner D, Oliveira M, et al. A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross-resistance to non-nucleoside reverse transcriptase inhibitors. *AIDS*. 2003;17:F1–F5.
6. The Thai Working Group on HIV/AIDS Projection. Projections for HIV/AIDS in Thailand: 2000–2020. 2001. Karnsana Printing Press, Bangkok.
7. Pathipvanich P, Ariyosh K, Rojanawiwat A, et al. Survival benefit from non-highly active antiretroviral therapy in a resource-constrained setting. *J Acquir Immune Defic Syndr*. 2003;32:157–160.
8. Kanshana S, Simond RJ. National program for preventing mother-child HIV transmission in Thailand: successful implementation and lessons learned. *AIDS*. 2002;16:953–959.
9. Hecht FM, Grant RM, Petropoulos CJ, et al. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. *N Engl J Med*. 1998;339:307–311.
10. Yerly S, Kaiser L, Race E, et al. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet*. 1999;354:729–733.
11. Salomon H, Wainberg MA, Brenner B, et al. Prevalence of HIV-1 resistant to antiretroviral drugs in 81 individuals newly infected by sexual contact or injecting drug use. Investigators of the Quebec Primary Infection Study. *AIDS*. 2000;14:F17–F23.
12. Frenkel LM, Wagner LE 2nd, Demeter LM, et al. Effects of zidovudine use during pregnancy on resistance and vertical transmission of human immunodeficiency virus type 1. *Clin Infect Dis*. 1995;20:1321–1326.
13. Richman DD, Guatelli JC, Grimes J, et al. Detection of mutations associated with zidovudine resistance in human immunodeficiency virus by use of the polymerase chain reaction. *J Infect Dis*. 1991;164:1075–1081.
14. Eastman PS, Urdea M, Besemer D, et al. Comparison of selective polymerase chain reaction primers and differential probe hybridization of polymerase chain reaction products for detemination of relative amounts of codon 215 mutant and wild-type HIV-1 populations. *J Acquir Immune Defic Syndr*. 1995;9:264–273.
15. Rust S, Funke H, Assmann G. Mutagenically separated PCR (MS-PCR): a high specific one step procedure for easy mutation detection. *Nucleic Acids Res*. 1993;21:3623–3629.
16. Frater AJ, Chaput CC, Beddows S, et al. Simple detection of point mutations associated with HIV-1 drug resistance. *J Virol Methods*. 2001;93: 145–156.
17. Myint L, Ariyoshi K, Yan H, et al. Mutagenically separated PCR assay for rapid detection of M41L and K70R zidovudine resistance mutations in CRF01_AE (subtype E) human immunodeficiency virus type 1. *Antimicrob Agents Chemother*. 2002;46:3861–3868.
18. Birch C, Middleton T, Hales G, et al. Limited evolution of HIV antiretroviral drug resistance-associated mutations during the performance of drug resistance testing. *J Acquir Immune Defic Syndr*. 2003;32:57–61.
19. Decamps D, Flandre P, Joly V, et al. Effect of zidovudine resistance mutations on virologic response to treatment with zidovudine or stavudine, each in combination with lamivudine and indinavir. *J Acquir Immune Defic Syndr*. 2002;31:464–471.
20. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science*. 1989;246: 1155–1158.
21. Kellam P, Boucher CA, Larder BA. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. *Proc Natl Acad Sci U S A*. 1992; 89:1934–1938.
22. Kellam P, Boucher CAB, Tijanagel JMGH, et al. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. *J Gen Virol*. 1994;75:341–351.
23. UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance. Analysis of prevalence of HIV-1 drug resistance in primary infections in the United Kingdom. *BMJ*. 2001;322:1087–1088.
24. Hance AJ, Lemiale V, Izopet J, et al. Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. *J Virol*. 2001;75:6410–6417.
25. Chan K, Galli R, Montaner J, et al. Prolonged retention of drug resistance mutations and rapid disease progression in the absence of therapy after primary HIV infection. *AIDS*. 2003;17:1256–1258.