treated with suboptimum ARV drug therapy such as monotherapy or dual therapy. The effect of such regimens on mortality remains largely unknown in the developing countries where non-B subtype HIV-1 prevails. Here, we report mortality rates among HIV-1–infected patients attending a government referral hospital and the effect of ARV drug therapy.

PATIENTS AND METHODS

We conducted a retrospective cohort study at Lampang Hospital in northern Thailand. Lampang Hospital is a government referral hospital with approximately 800 beds, and it is situated in the center of Lampang province, a province 100 km to the south of Chiang Mai in northern Thailand. We surveyed all HIV-1–infected adult patients aged 16 years and older who attended the clinic at the Day Care Center (DCC) at Lampang Hospital from October 2, 1995, when the center was established, until October 31, 1999. Demographic, clinical, and laboratory data and history of ARV drug therapy were collected from hospital records. Clinical status at first visit was characterized as either with or without any HIV-related symptom, based on the Thai government's national guideline for the clinical management of HIV infection (5). Two designated nurses and one clinician, who made the clinical diagnosis in all cases, have run this clinic throughout the observation period. When their disease progression was clinically apparent, patients were often not tested for CD4 counts because of the hospital's limited financial resources. The survival status of patients until October 31, 1999 was ascertained from the hospital records, mailing letters, and death certificates at the Lampang Provincial Health Office. Data were coded twice, double-entered, and validated using the EpiInfo program. Survival of the patients was analyzed for specific variables, including age, sex, first CD4 count, year of registration, clinical status at first visit to the DCC, and use of antiretroviral drug(s). The Cox proportional hazard model was applied for the survival analysis using STATA 6.0.

RESULTS

A total of 1110 HIV-1 patients attended the clinic during the study period: 383 (34.5%) patients were female, the median (range) age was 30.0 (16–69) years old, the majority (96.9%) contracted HIV through heterosexual contact, and 778 (70.1%) were symptomatic. CD4 counts within 1 year of the first visit were available in 681 patients; the majority of patients (93.7%) were tested for CD4 counts within 6 months of their first visit. Four hundred fourteen patients were not tested for CD4 counts throughout the study period. Fifteen patients who were tested for CD4 counts, but not within 1 year of their first visit, were excluded from the analysis. The median (interquartile range [IQR]) interval from the first visit to the CD4 test was +5 (+2–+28) days for untreated patients, +7 (−142, +64) days for patients who received ARV drug monotherapy, and +7 (+2–+23) days for patients who received ARV drug dual therapy. The median (IQR) CD4 count was 90 (25–290) cells/μL; 348 patients had already progressed into the lowest CD4 group of <100 cells/μL, whereas 257 patients had a CD4 count lower than 50 cells/μL.

Two hundred fifty-five patients (23%) received ARV drug therapy during the observation period: 79 patients were treated with an ARV drug as monotherapy (69 patients were treated with zidovudine [AZT] alone and 10 patients were treated with didanosine [ddI] or zalcitabine [ddC] alone); 130 patients were treated with AZT and either ddI or ddC as dual therapy (19 patients initially treated with AZT alone but subsequently receiving ddI or ddC as dual therapy were categorized in the dual therapy group); 19 patients were treated with triple therapy, including a protease inhibitor; and 27 patients were treated with AZT and either ddI or ddC, but only one drug was administered at a time. The 46 patients of the last two treatment groups were excluded from the survival analysis because of their small number. Median (IQR) interval from the first visit to the starting date of ARV drug therapy was significantly shorter among patients with monotherapy than among patients with dual therapy [31.5 (0–106) and 76 (8–403) days, respectively; $p < .001$ by Kruskal-Wallis test]. Median (IQR) duration of ARV drug therapy in patients treated with monotherapy was 211 (140.5–310) days. Among patients treated with dual therapy, median (IQR) duration of ARV therapy was 367 (235–388) days.

Follow-up duration was measured from the date of the first visit for untreated patients and from the starting date of ARV drug therapy for treated patients until death or October 31, 1999, or the date patients were last seen alive if their survival status on October 31, 1999 was not known. Of the total of 1110 patients, 29 (2.6%) did not have any follow-up information, and these patients were excluded from the analysis. We had data on duration of follow-up for the remaining 1081 (97.4%) patients. During the observation period, 607 (56.2%) patients died, and 354 (32.7%) patients were confirmed to be alive until October 31, 1999. One hundred twenty (11.1%) patients whose status on October 31, 1999 was not known, were right-censored when last seen to be alive. The median (IQR) follow-up duration was 271 (112–534) days, and there were a total of 1175 person-years of observation (PYOs). Results of the survival analysis are summarized in Table 1. Our study revealed a strikingly high rate of mortality: the mortality rate per 100 PYOs in the lowest CD4 group of <100 was 79.6%; it was over 100% in the group without available CD4 data. Sex, age group, registered year, clinical status, CD4 group, and ARV drug group were all significantly related to death in the univariate analysis, but the association with sex and age group was not significant in the multivariate analysis. Figure 1 shows a Kaplan-Meier survival curve strati-

TABLE 1. *Mortality rates of HIV-1–infected patients by risk factors*

Group	Patients, *n*	Deaths/PYO	Mortality rate per 100 PYO (95% CI)	Adjusted hazard ratio (95% CI)	*p* value for variable from multivariate (univariate) analysis
Sex					
Male	705	483/651	74.2 (67.6, 80.8)	1.0	
Female	376	124/524	23.6 (19.5, 27.8)	0.81 (0.66, 1.00)	.060 (<.001)
Age group					
<25 years	167	79/220	35.9 (28.0, 43.8)	1.0	
≥25 years	914	528/955	55.3 (50.6, 60.0)	1.18 (0.92, 1.52)	.188 (.003)
Registered year					
1995–1998	943	588/1126	52.2 (48.0 56.4)	1.0	
1999	138	19/49	38.9 (21.4, 56.3)	0.57 (0.36, 0.90)	.016 (.012)
Clinical status					
Asymptomatic	324	54/542	10.0 (7.3, 12.6)	1.0	
Symptomatic	757	553/633	87.3 (80.1, 94.6)	3.76 (2.72, 5.21)	<.001 (<.001)
CD4 group					
≥500	60	2/103	1.9 (0, 4.7)	1.0	
200–499	192	26/338	7.7 (4.7, 10.7)	4.33 (1.03, 18.3)	.046 (.054)
100–199	77	27/125	21.6 (13.5, 29.8)	6.27 (1.47, 26.8)	.013 (.001)
0–99	345	252/317	79.6 (69.7, 89.4)	15.42 (3.8, 63.3)	<.001 (<.001)
Not available	392	293/256	114.5 (101.4, 127.6)	24.44 (6.0, 99.9)	<.001 (<.001)
Antiretroviral drug					
None	828	501/748	67.0 (61.1, 72.9)	1.0	
Mono	78	52/127	41.0 (29.9, 52.2)	0.65 (0.48, 0.87)	.004 (.105)
Dual	129	31/204	15.2 (9.8, 20.5)	0.43 (0.29, 0.62)	<.001 (<.001)

PYO, person-years of observation.

fied by ARV drug group among patients with a low CD4 count of < 200 cells/μL and patients with a high CD4 count of <200 cells/μL. Patients who were treated with dual therapy had a clear advantage in survival over patients treated with monotherapy or patients without any ARV drug. The effect of ARV drug therapy on mortality was quantitatively analyzed by calculating a hazard ratio after adjusting for sex, age group, years of registration, clinical status at first visit, and CD4 group (Table 1). The adjusted hazard ratio of monotherapy to no therapy was 0.65, and that of dual therapy was 0.43.

DISCUSSION

Although this study was conducted retrospectively, the high rate of survival follow-up enabled us to estimate the mortality rate of patients attending a government hospital in northern Thailand. Our study was hospital based; thus, the patient population was inevitably biased toward advanced disease stage. Nevertheless, the majority of patients had CD4 data, and we could evaluate mortality in CD4 stratified groups. When we assumed that the mortality rate was constant, 9.5 per 100 PYOs of patients with a high CD4 count of ≥500 cells/μL, 38.5 per 100 PYOs of patients with a CD4 count of 200 to 499 cells/μL, and over 100 per 100 PYOs of patients with a low CD4 count <200 cells/μL were estimated to die within 5 years. These results are compatible with the data obtained from a prospective cohort of female commercial sex workers in northern Thailand (3). It was interesting to find that patients who registered at the DCC in 1999 or after died less frequently than patients who registered there before 1999. Because the number of newly registered patients per year peaked in 1996, it constantly declined and the proportion of female patients increased (data not shown). The association remained significant, even after adjusting for all other factors. Further investigation is needed.

To our knowledge, this is the first report showing that suboptimal ARV drug regimens had a substantial survival benefit in developing countries like Thailand, where non-B subtype HIV-1 of CRF01_AE prevails (6): the relative reduction in mortality compared with no therapy was 35% by monotherapy and 57% by dual therapy. Our results were similar to the findings of previous studies in Western countries (7,8). The cost of dual therapy, including generic AZT, was 3.5 times less expensive than triple therapy with ritonavir at the time we closed this study. As long as the gap between the cost for suboptimal therapy and highly active antiretroviral treatment (HAART) is immense, clinicians working in resource-limited countries will continue to face the dilemma of treating a smaller number of patients with optimal therapy or a larger number with suboptimal therapy. Prevailing drug-resistant viruses are one of the major concerns of dual therapy. Nevertheless, most patients receiving ARV drug therapy were not in an active high-risk group and were less likely to have induced a

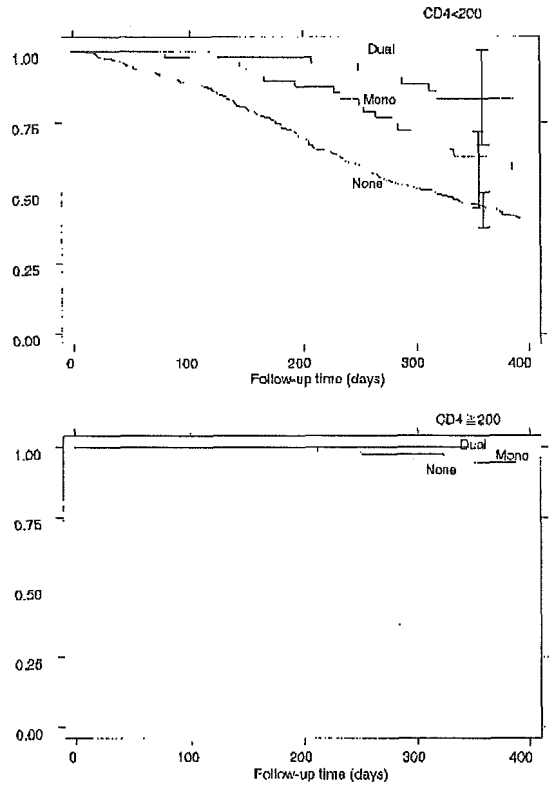

FIG 1. Kaplan-Meier survival curves for survival stratified by antiretroviral drug groups among patients with a CD4 count <200 cells/μL (*top panel*) and among patients with a CD4 count ≥200 cells/μL (*bottom panel*). Vertical bars in top panel show 95% CIs at 1 year of follow-up.

new infection. Reducing the effectiveness of future triple therapy among patients exposed to dual therapy is another concern. Nevertheless, if not treated with any ARV drug, most patients with a low CD4 count will soon die. Since mid-2002, the hospital has been making the transition from dual therapy to a less expensive generic medicine called "GPOvir," which is a combination of the three drugs stavudine (d4T), lamivudine (3TC), and nevirapine. Further studies are required to reveal the survival benefit of GPOvir in CRF01_AE-infected patients. It would be difficult to evaluate the superiority of GPOvir over dual therapy without data on dual therapy. Many clinicians working in developing countries will continue to face the frustration of limited drug choices. A further cost reduction for other HAART therapy is also impatiently awaited.

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Impaired Processing and Presentation of Cytotoxic-T-Lymphocyte (CTL) Epitopes Are Major Escape Mechanisms from CTL Immune Pressure in Human Immunodeficiency Virus Type 1 Infection

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Investigating escape mechanisms of human immunodeficiency virus type 1 (HIV-1) from cytotoxic T lymphocytes (CTLs) is essential for understanding the pathogenesis of HIV-1 infection and developing effective vaccines. To study the processing and presentation of known CTL epitopes, we prepared Epstein-Barr virus-transformed B cells that endogenously express the *gag* gene of six field isolates by adopting an *env/nef*-deletion HIV-1 vector pseudotyped with vesicular stomatitis virus G protein and then tested them for the recognition by Gag epitope-specific CTL lines or clones. We observed that two field variants, SLFNTVAVL and SVYNTV ATL, of an A*0201-restricted Gag CTL epitope SLYNTVATL, and three field variants, KYRLKHLVW, QYRLKHIVW, and RYRLKHLVW, of an A24-restricted Gag CTL epitope KYKLKHIVW escaped from being killed by the CTL lines, despite the fact that they were recognized when the synthetic peptides corresponding to these variant sequences were exogenously loaded onto the target cells. Thus, their escape is likely due to the changes that occur during the processing and presentation of epitopes in the infected cells. Mutations responsible for this mode of escape were located within the epitope regions rather than the flanking regions, and such mutations did not influence the virus replication. The results suggest that the impaired antigen processing and presentation often occur in HIV-1 field isolates and thus are one of the major mechanisms that enable HIV-1 to escape from CTL recognition. We emphasize the importance of testing HIV-1 variants in an endogenous expression system.

Accumulated evidence has indicated a critical role of cytotoxic T lymphocytes (CTLs) in controlling human immunodeficiency virus (HIV) replication during acute and chronic infection (16). Eliciting HIV type 1 (HIV-1)-specific CTLs has been thought to be crucial for effective HIV/AIDS vaccines (15). However, despite the presence of CTLs, the majority of HIV-1-infected cases eventually progress to AIDS, probably as a consequence of the emergence of escape mutants from CTLs (8, 20). Among immunized monkeys, which developed strong cellular immune responses against HIV-1, eventual vaccine failure occurs by viral escape from CTLs (2). Thus, investigating the mechanisms of CTL failure to control the virus is essential to understanding the pathogenesis of HIV-1 infection and to develop HIV/AIDS vaccines.

The high rate of HIV-1 replication in vivo indicates that HIV-1 has tremendous ability to mutate swiftly (9, 30) and to make a dynamic adaptation to host-immune environments (3, 14, 18, 21, 31). Several mutations have been described in CTL epitopes in HIV-1-infected individuals, which result in either a lack of binding to the MHC class I molecule or nonrecognition by T-cell receptor (TCR) (3, 8, 12, 20, 21). Consequently, the virus escapes from CTL recognition. There are other mutations that do not lead to either escape effects (12); very little is known about the influence of these mutations on CTL recognition. CTL antigens are processed and presented on the cell surface in a very complex manner. Peptides are cleaved from endogenously synthesized proteins by proteasome in the cytoplasm and transported into the endoplasmic reticulum by the transporter of antigen presentation. Amino-terminal extended peptides are trimmed to the right size of peptides by aminopeptidases, which exist in both the cytoplasm and the endoplasmic reticulum (23). These steps have various degrees of substrate sequence specificity (17). The generated peptides should have sufficient affinity to bind to a major histocompatibility complex (MHC) class I molecule in the presence of various other peptides derived from host proteins and to maintain the stability of peptide-MHC complexes until they are presented on the cell surface (28). Thus, it is plausible that some amino acid substitutions in the epitope and its flanking regions have a significant influence on antigen processing and presentation. In the present study, we hypothesized that such mutations often enable HIV-1 to escape from CTL recognition.

Conventionally, the intracellular HIV-1 antigen processing and presentation has been studied with recombinant vaccinia viruses expressing an HIV-1 gene (3, 4, 11, 20, 26). Several

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TABLE 1. Characteristics of five HIV-1-infected donors[a]

Donor	HLA type			CD4 count/μl	Virus load (copies/ml)	No. of isolated clones
	A	B	Cw			
IMS1	*0201/2402	52/75	3	286	<400	3
IMS2	*0201/31	27/5101	2	797	<400	2
IMS4	*0207/2402	46/52	1	448	<400	2
IMS6	2402/26	7/5101	7	368	3.6×10^5	3
IMS7	1/−	37/−	6	544	1.3×10^3	3

[a] HLA alleles, CD4 count, viral load, and the number of isolated clones from each donor's sample are shown.

studies have addressed this issue in the context of HIV-infected T cells (4, 29, 32, 33). Most studies, however, have only evaluated a single or a few laboratory-established strains. The CTL recognition of HIV-1 clinical isolates has been evaluated, in most cases, by exogenously applying synthetic variant peptides to the cell surface to replace MHC-bound peptides (8, 12, 20, 21). Very little is known about how the antigenic products of HIV-1 clinical isolates are processed and presented in the infected cells. To address this issue, we prepared CTL target cells that endogenously express the *gag* gene derived from HIV-1 clinical isolates by adopting an *env/nef*-deletion HIV-1-based vector pseudotyped with vesicular stomatitis virus protein G (VSV-G) proteins. Here, we show evidence that HIV-1 escapes from CTL recognition often via the impairment of antigen processing and presentation.

MATERIALS AND METHODS

Subjects. Peripheral blood mononuclear cells (PBMC) were collected from five HIV-1-infected individuals from the HIV clinic affiliated with the Institute of Medical Science, University of Tokyo. Two individuals (IMS1 and IMS2) had no therapy; one individual (IMS6) was off drugs but had received treatment (zidovudine alone) 2 years prior to blood sampling; two individuals (IMS4 and IMS7) had received therapy (zidovudine-lamivudine-indinavir and stavudine-lamivudine-nelfinavir, respectively) but for less than 3 months. CD4 count, viral load, and HLA type of the recruited individuals are shown in Table 1. HLA class I typing was initially performed by serology. Subtyping of HLA-A2 was done by a PCR–sequence-specific primer method (Dynal Classic SSP HLA-A2; Dynal A.S., Oslo, Norway).

Isolation and cloning of full-length *gag*. Full-length *gag* was amplified from proviral DNA extracted from the PBMC by nested PCR with *Pfu* DNA polymerase (Strategene, La Jolla, Calif.) and oligonucleotides specific for HIV-1 long terminal repeat (LTR) and reverse transcription (RT) regions. Four oligonucleotides were mixed as outer primers: the sense primers 1U5AS-S (5′-ACTCTG GTADCTAGAGATCCCTCA-3′; the position in HXB2 being 578 to 601) and TAR-2 (5′-TGAGCCTGGGAGCTCTCTGGCT-3′; 478-499) and the antisense primers RT7A-A (5′-TATGTTGAYAGGTGTAGGTC-3′; 2485 to 2504) and RT18A-A (5′-CTACYARTACTGTACCTATAG-3′; 2464 to 2484). Two oligonucleotides were used as inner primers: the sense primer TPBS1-S (5′-AAAA TCTCTAGCAGTGGCGCCCGAACAGG-3′; the position in HXB2 being 622 to 650) and the antisense inner primer PRO6A (5′-ACTGTATCATCTGCTCC TGTRTCTAA-3′; 2322 to 2347). The thermocycling conditions were 95°C for 45 s, 50°C for 45 s, and 72°C for 210 s (30 cycles) and 72°C for 7 min for both primary and secondary PCR. The PCR products were purified by using spin columns (QIAquick PCR purification kit; Qiagen, Santa Clarita, Calif.) and cloned into PT7Blue3 vector by using a commercial cloning kit (Perfectly Blunt cloning kit; Novagen, Dedham, Mass.). Two to three clones for each individual were sequenced by an automated sequencer (ABI Prism 377 automated DNA sequencer; Perkin-Elmer, Norwalk, Conn.) with BigDye terminators (PE Applied Biosystems, Foster City, Calif.). The sequences of *gag* clones that were used in the present study are available under GenBank Accession numbers as follows: AB074049 (IMS1-28), AB074050 (IMS1-29), AB074052 (IMS2-5), AB074058 (IMS4-24), AB074061 (IMS6-34), and AB074064 (IMS7-11).

Construction of HIV-1 vector. The design of HIV-1 vector, pCTLpac, is shown in Fig. 1. The backbone of the vector is derived from an infectious molecular clone, HXB2Ecogpt (22), which lacks the function of *vpr*, *vpu*, and *nef* genes. We deleted a 1.5-kb portion from the *env*-coding region but kept the function of Rev responsive element, Tat, and Rev. The *nef* gene was replaced with the puromycin *N*-acetyltransferase (*pac*) gene (pPUR; BD Biosciences Clontech, Palo Alto, Calif.) by using *Xho*I and *Cla*I sites where the *Cla*I site was introduced by site-directed mutagenesis. *Sbf*I and *Swa*I sites were introduced by site-directed mutagenesis in the upstream of the *gag* (nucleotide 788) and in the *pol* (nucleotide 3717), respectively. The fragment from *Spe*I in the gag (nucleotide 1507) to the *Swa*I was then replaced with that of a previously published vector, pHXB2cv (25), which has a *Not*I site but lacks an *Sbf*I site in the *pol* gene. Consequently, the final construct carries the single *Sbf*I site (nucleotide 788) and the *Not*I site (nucleotide 2275) that corresponds to the 10th codon of protease. These sites were used for incorporating the *gag* clones derived from clinical isolates into the pCTLpac vector. We confirmed that the expected variant sequences were inserted in the vector by sequencing.

Generation of VSV-G pseudotype virus. Subconfluent COS7 cells in 25-cm^2 T flasks (Becton Dickinson, Lincoln Park, N.J.) were cotransfected with 4 μg of pCTLpac and 2 μg of pVSVG (BD Biosciences Clontech), which expresses VSV-G protein, by lipofection (FuGENE6; Roche Molecular Biochemicals, Mannheim, Germany) and then incubated for 48 to 60 h. The supernatant, which contains pseudotype viruses carrying the HIV-1 vector with VSV-G envelope proteins, was harvested, filtered through a 0.45-μm (pore-size) Millex filter (Millipore, Bedford, Mass.), and used as pseudotype virus stocks, some of which were stored at −80°C before use. The amount of p24 antigen in the stocks was measured by p24 antigen capture enzyme-linked immunosorbent assay (ELISA; RETRO-TEK; Zeptometrix Corp., Buffalo, N.Y.). The range of the p24 antigen yield was 40 to 100 ng/ml.

Preparation of target cells by using VSV-G pseudotyped HIV-1 vector. Epstein-Barr virus-transformed B-lymphocyte lines (B-LCLs) were infected with pseudotype virus stocks for 6 h at 37°C. The medium was then replaced with fresh RPMI 1640 (Sigma-Aldrich, St. Louis, Mo.) supplemented with 10% fetal bovine serum (R10; HyClone, Logan, Utah), and the cells were incubated for an additional 36 h. Subsequently, 0.5 μg of puromycin (BD Biosciences Clontech)/ml was added to the R10 medium to select transduced cells. The culture was maintained until the number of transduced cells became sufficient for CTL experiments. When 10^6 B-LCLs were infected with 1 ml of pseudotype virus stocks, the transduction efficiency was 20 to 30%. Usually, more than 10^7 transduced cells were generated within 2 weeks and used as CTL target cells.

To standardize the expression level of Gag protein in target cells, we quantified the amount of extracellular p24 antigen that 10^6 cells per ml of target cells had produced in 24 h. The supernatant was harvested before (supernatant A) and after (supernatant B) the 24 h of culture for the measurement of p24 antigen by p24 antigen capture ELISA (Zeptometrix Corp.). The level of p24 antigen production was defined by the difference in the concentration of p24 antigen between supernatants A and B. If the target cells produced p24 antigen that was >1 ng/ml in 24 h, they were used for CTL experiments, since the specific percent lysis did not significantly differ among target cells producing Gag protein above

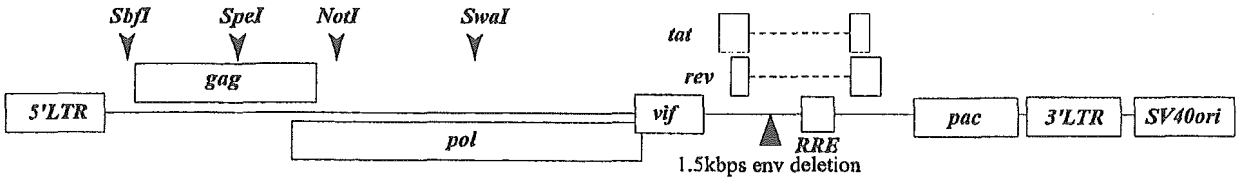

FIG. 1. Structure of pCTLpac. A 1.5-kbp portion of *env* was deleted (▲). Puromycin *N*-acetyltransferase gene (*pac*) was inserted in the *nef* region. The locations of restriction enzyme sites are indicated (▼). RRE, Rev responsive element.

this level (data not shown). We also investigated the level and pattern of protein expression of *gag* variants by Western blot analysis, as previously described (25).

^{51}Cr release experiments with HLA-class I-mismatched target cells in parallel with HLA-class I matched target cells of different donors confirmed that these target cells were recognized by CTLs in an HLA-restricted manner (data not shown). Repeated experiments showed that specific lysis of blank controls was equivalent to that of cells expressing *gag* variants that are known to escape either from TCR recognition or MHC binding. Some examples appear in the Results section below: specific lysis against IMS2-5 (Fig. 3a), IMS4-24 (Fig. 3c), IMS6-34 (Fig. 3e), and HXB2-wild (Fig. 5b). Thus, we regarded the blank control as a negative control.

Preparation of target cells by using recombinant vaccinia viruses. Recombinant vaccinia viruses used in the experiment shown in Fig. 3b were made as previously described (10). HLA-matched B-LCLs were infected with recombinant vaccinia viruses at a multiplicity of infection of 3:1 overnight before being tested in a ^{51}Cr release assay.

Effector cells. Peptide-specific CTL lines were induced from PBMC of HIV-1-infected donors. Half of the PBMC were stimulated with phytohemagglutinin (2 μg/ml) for 24 h and then pulsed with corresponding peptides at 100 μM for 1 h and irradiated before being added to the other half of the PBMC. A total of 3×10^5 cells in each well of a 96-well U-bottom plate, with at least 10 wells for each sample, were cultured in R10; 10% Lymphocult T (Biotest, Dreieich, Germany) was added to the medium on day 3 of culture. The CTL lines were maintained by adding fresh R10 medium containing 10% Lymphocult T every 3 to 4 days and splitting the well accordingly. Assays were performed on day 14 to 28 of culture.

Synthetic peptides. Peptides were manufactured at the Takara Shuzo Co., Ltd. (Shiga, Japan). The purity of peptides was >99% as determined by high-pressure liquid chromatography, and the identity of peptides was confirmed by matrix-assisted laser desorption ionization–mass spectrometry. Lyophilized peptides were dissolved in dimethyl sulfoxide and diluted in phosphate-buffered saline to make a stock concentration (2 mM). Further dilution was made in RPMI 1640 to make working concentrations of 200 μM for the induction of CTLs and of 20 μM for the preparation of target cells.

^{51}Cr release assay. In 96-well U-bottom plates, target cells were divided into aliquots at 5,000 per well. Effector cells were added to target cells at different effector/target (E:T) ratios. The amount of ^{51}Cr release in the culture supernatants was quantified after 6 h of incubation, and the percent specific lysis was determined by using the following formula: $[(E - M)/(D - M)] \times 100$, where E is the experimental ^{51}Cr release, M is the ^{51}Cr released in the presence of culture medium (which ranged between 15 and 25% of total release), and D is the total ^{51}Cr released in the presence of 5% Triton X-100 detergent. The results were regarded as positive when recognition of the HIV target was >10% above the control. The SD_{50} is the peptide concentration giving 50% of maximal specific lysis of target cells pulsed with 10 μM synthetic peptide (28).

Replication kinetics assay. Subconfluent 293T cells in Falcon 25-cm^2 T flasks (Becton Dickinson) were transfected by lipofection (Roche Molecular Biochemicals), with 2 μg of HXB2cv replication-competent HIV-1 plasmids, in which various mutations were introduced. After 60 h of culture, the supernatant was harvested, filtered through a 0.45-μm-pore-size filter, and used as mutant virus stocks. Two million Jurkat cells or eight million H9 cells were infected with an equivalent of 40 ng of p24 antigen of mutant viruses in 2 ml of R10 for 1 h. Cells were washed three times with 10 ml of R10, resuspended with 5 ml of R10, and cultured in a 12.5-cm^2 T flask at 37°C in 5% CO_2. Every 2 or 3 days, 1.5 ml of supernatant was harvested and replaced with fresh R10. The concentration of p24 was measured by using a p24 ELISA kit (Zeptometrix Corp.).

This study was approved by the Ethics Committee of the University of Tokyo.

RESULTS

Full-length *gag* clones of field isolates. We used 6 of 13 full-length *gag* clones that were isolated from the five infected individuals (Fig. 2). All of the clones did not have any stop codons. In the present study, we focused on the processing and presentation of three CTL epitopes: the HLA-A*0201-restricted epitope SLYNTVATL, the A24-restricted epitope KYKLKHIVW in p17 matrix protein (MA), and the HLA-B*5101-restricted epitope NANPDCKTI in p24 capsid protein (CA) (11, 26, 27). Amino acid sequences within the three epitope regions and the N- and C-terminal 15-amino-acid residues flanking each epitope were analyzed; the six clones were selected to maximize the diversity of amino acid sequences in the epitopes and its flanking regions.

The A*0201-restricted epitope and its flanking regions were highly variable. However, we did not observe a previously recognized variation in the flanking region, Arg (R) to Lys (K) at position 76 in our clones (4). In contrast, the B*5101-restricted epitope and its flanking regions were conserved except for clones IMS2-5 and IMS4-24. In the A24-restricted epitope and its flanking regions, variations were seen almost exclusively within the epitope region with two exceptions, a Lys (K)-to-Ser (S) mutation at position 26 (K26S) in clone IMS4-24 and an Arg (R)-to-Lys (K) at position 15 in clone IMS2-5. The Lys (K)-to-Arg (R) mutation at position 30 within the A24-restricted epitope was seen more frequently than any other sequences; none of the 13 clones had the wild-type sequence of KYKLKHIVW. We incorporated the six *gag* clones into the HIV-1 vector with *env* and *nef* deleted, pCTLpac (Fig. 1), to make target cells expressing *gag* genes of these filed isolates.

CTL recognition of target cells endogenously expressing *gag* genes of clinical isolates. We generated A*0201-restricted SLYNTVATL (wild type) epitope-specific oligoclonal CTL lines from one HIV-1-infected individual (IMS1) with A*0201 and used the lines to test the killing of the six different *gag* clones expressed on A*0201-matched B-LCLs by a conventional ^{51}Cr release assay. The A*0201-restricted CTLs efficiently recognized target cells expressing *gag* clones IMS1-29, IMS1-28, and IMS6-34, which encode either wild type or the SLYNTIATL sequence in the CTL epitope region. In contrast, the same CTLs did not recognize cells expressing *gag* clones IMS2-5, IMS4-24, and IMS7-11, which encode SLYNLVATL, SLFNTVAVL, and SVYNTVATL, respectively, indicating that these clones escaped from A*0201-restricted CTL recognition (Fig. 3a).

CTL recognition of IMS1-29 and IMS6-34 was also tested with recombinant vaccinia viruses expressing the *gag* gene of these variants in parallel with the VSV-G-pseudotyped HIV-1 vectors. The HIV-1 vector method demonstrated the CTL killing as well or slightly better than the vaccinia method did (Fig. 3b).

We used three B*5101-restricted NANPDCKTI -specific CTL clones to test the CTL recognition of five representative *gag* clones. The CTL clones recognized four *gag* clones, which convey the wild-type B*5101-restricted epitope sequence; they also recognized IMS2-5 that had a substitution in the flanking region. None of the clones recognized the IMS4-24 clone, which had the variant sequence NSNPDCKNI in the epitope region (Fig. 3c).

A24-restricted KYKLKHIVW (wild type) specific-CTL lines did not recognize synthetic peptides of the most common sequence, KYRLKHIVW (3R mutant type), and the other variant, RYRLKHIVW (Fig. 3d). These two variants were shown to bind to the A*2402 MHC class I molecule in a binding assay (data not shown). We screened eight A24-positive individuals for the presence of CTL activities against the 3R mutant epitopes and found one individual who carried CTLs recognizing the 3R mutant peptide. A24-restricted 3R mutant-reactive CTL lines were induced from this A24-positive individual and used for the remaining experiments. The 3R mutant-reactive CTL lines recognized target cells expressing IMS1-29 and IMS4-24 *gag* clones, both of which carry the 3R mutant sequence, but did not recognize any

(1) A*0201-restricted epitope (amino acid 62-100)

origin	62							70							80							90							100
HXB2	G	Q	L	Q	P	S	L	Q	T	G	S	E	L	E	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q
IMS 1-28	E	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	V
IMS 1-29	E	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V
IMS 2-5	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	K
IMS 4-24	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	V	-	-	-	-	-	K
IMS 6-34	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V
IMS 7-11	A	-	-	H	-	A	-	K	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-					

origin	90							100
HXB2	I	E	I	K	D	T	K	E A
IMS 1-28	-	-	-	-	-	-	-	- -
IMS 1-29	-	-	-	-	-	-	-	- -
IMS 2-5	-	-	-	-	-	-	-	- -
IMS 4-24	-	-	-	-	-	-	-	- -
IMS 6-34	-	-	-	-	-	-	-	- -
IMS 7-11	-	-	-	-	-	-	-	- -

(2) B51 restricted epitope (amino acid 310-348)

origin	310									320										330										340							348
HXB2	S	Q	E	V	K	N	W	M	T	E	T	L	L	V	Q	N	A	N	P	D	C	K	T	I	L	K	A	L	G	P	A	A	T	L	E	E	M	M	T
IMS 1-28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 2-5	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 4-24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 6-34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 7-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(3) A24-restricted epitope (amino acid 13-51)

origin	13									20										30										40									51
HXB2	L	D	R	W	E	K	I	R	L	R	P	G	G	K	K	K	Y	K	L	K	H	I	V	W	A	S	R	E	L	E	R	F	A	V	N	P	G	L	L
IMS 1-28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 2-5			K													Q	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 4-24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 6-34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 7-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	R	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 2. Sequence variation in three CTL epitopes and their flanking regions. The amino acid sequences of six *gag* clones are shown. The reference sequence is derived from HXB2, and the differences are indicated. The numbering is done according to the HIV sequence database, Los Alamos National Laboratory, Los Alamos, N.Mex. The CTL epitope regions are boxed.

other target cells expressing different variants (Fig. 3e). Interestingly, IMS4-24 with Lys (K)-to-Ser (S) mutation at position 26 outside the epitope region was less well recognized than IMS1-29. We consistently observed this phenomenon in repeated experiments (data not shown).

CTL recognition of exogenously loaded variant peptides. To investigate whether the above findings of escape phenomenon from CTL killing were due to either loss of peptide binding to the MHC class I molecule or to the lack of TCR recognition, we prepared synthetic peptides that represented the variant epitopes and tested them for cross-recognition of the peptides in peptide titration assays by using the same CTL lines or clones that were used in experiments described for Fig. 3. To our surprise, A*0201-restricted CTL lines recognized the peptides of two A*0201-restricted CTL epitope variants, SVYNT VATL and SLFNTVAVL, which were not recognized by the CTLs when expressed endogenously. They recognized the SLFNTVAVL peptide less efficiently, with an SD_{50} of >100 nM (Fig. 4a). Target cells pulsed with SLYNLVATL peptide representing clone IMS 2-5 were not cross-recognized even at a saturated concentration (10 μM) (data not shown).

We also obtained similar discordant results in experiments of A24-restricted CTL epitope variants. A24-restricted 3R mutant-specific CTL lines recognized peptides of three variant s—KYRLKHLVW, RYRLKHLVW, and QYRLKHIVW—that were not recognized by the CTLs when they were expressed endogenously. In fact, the CTLs recognized QYRLKHIVW peptide even better than the 3R mutant peptide but did not cross-recognize the QYKLKHIVW peptide (Fig. 4b).

We tested one B*5101 variant peptide, NSNPDCKNI, in a peptide titration assay. This variant was not cross-recognized by any of the CTL clones even at a high concentration (1 μM) (Fig. 4c). The two amino acid mutations in this epitope coincided with two anchor residues to the MHC biding, suggesting that the lack of recognition of this variant was likely due to loss of peptide binding.

Mutations responsible for impairing the epitope processing and presentation. The discrepancies seen above between the CTL recognition of endogenously expressed and exogenously loaded antigen indicate that some mutations have caused the impairment of epitope processing and presentation. To locate specific variations that were responsible for the poor recognition of endogenously expressed HIV-1 *gag* variants, we constructed four different target vectors: an HXB2 *gag* sequence with A*0201-restricted epitope variations (SLFNTVAVL [HXB2-3F8V] or SVYNTVATL [HXB2-2V]) and IMS4-24- or IMS7-11-derived *gag* sequence with the wild-type A*0201 epitope sequence (IMS4-24-wild or IMS7-11-wild, respectively). The replacement of the variant epitope region with the

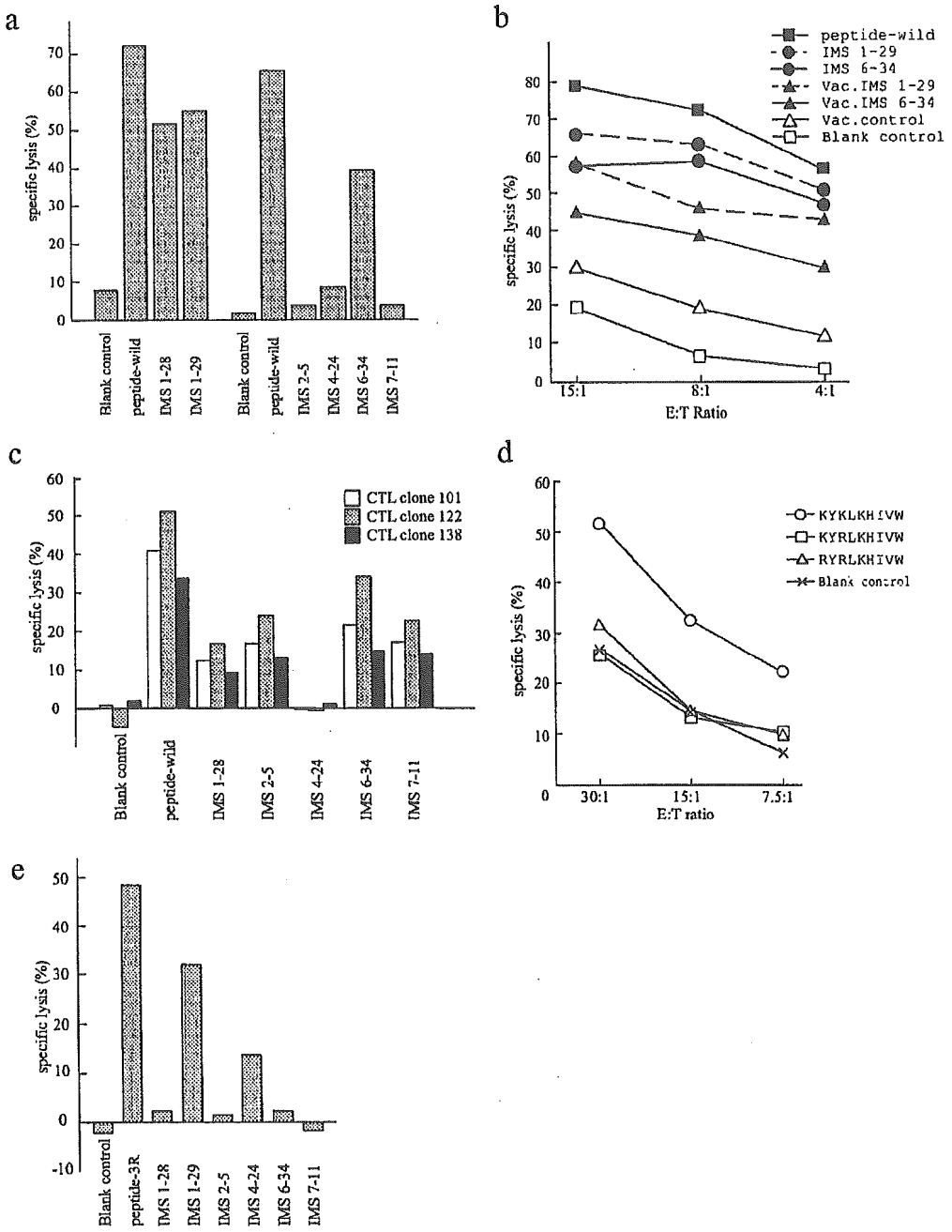

FIG. 3. (a) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) producing Gag proteins of clinical isolates. Peptide target cells were pulsed with the A*0201 wild-type peptide, SLYNTVATL (10 μM). A*0201-restricted SLYNTVATL -specific CTL lines were induced from a single donor (IMS1). The E:T ratio was 10:1. This experiment was repeated, with a different B-LCLs (HLA-A*0201/31 and HLA-B27/*5101), giving the same pattern of recognition (data not shown). (b) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) expressing *gag* clones of two clinical isolates with the VSV-G-pseudotyped HIV-1 vector versus recombinant vaccinia viruses. Recombinant vaccinia virus expressing the human CD4 gene was used as a vaccinia virus control (1). The effector and peptide target cells were prepared as described for panel a. (c) Specific lysis of B*5101-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) producing the Gag proteins of five clones. Three B*5101-restricted NANPDCKTI -specific CTL clones were used as effector cells at an E:T ratio of 2:1 (23). The peptide target was pulsed with the B51 wild-type peptide NANPDCKI (1μM). (d) Specific lysis of A24-matched B-LCLs (HLA-A24/− and HLA-B46/52) pulsed with the peptides KYKLKHIVW, KYRLKHIVW, and RYRLKHIVW at 10 μM. A24-restricted, KYKLKHIVW -specific CTL lines were induced from one A24-positive donor. (e) Specific lysis of A24-matched B-LCLs (HLA-A24/− and HLA-B46/52) producing variant Gag proteins. A24-restricted KYRLKHIVW (3R)-specific CTL lines were induced from another A24-positive donor. The peptide target was pulsed with 3R mutant type peptide (10 μM). The E:T ratio was 20:1. The lysis of target cells without any peptide pulsing is shown as a blank control.

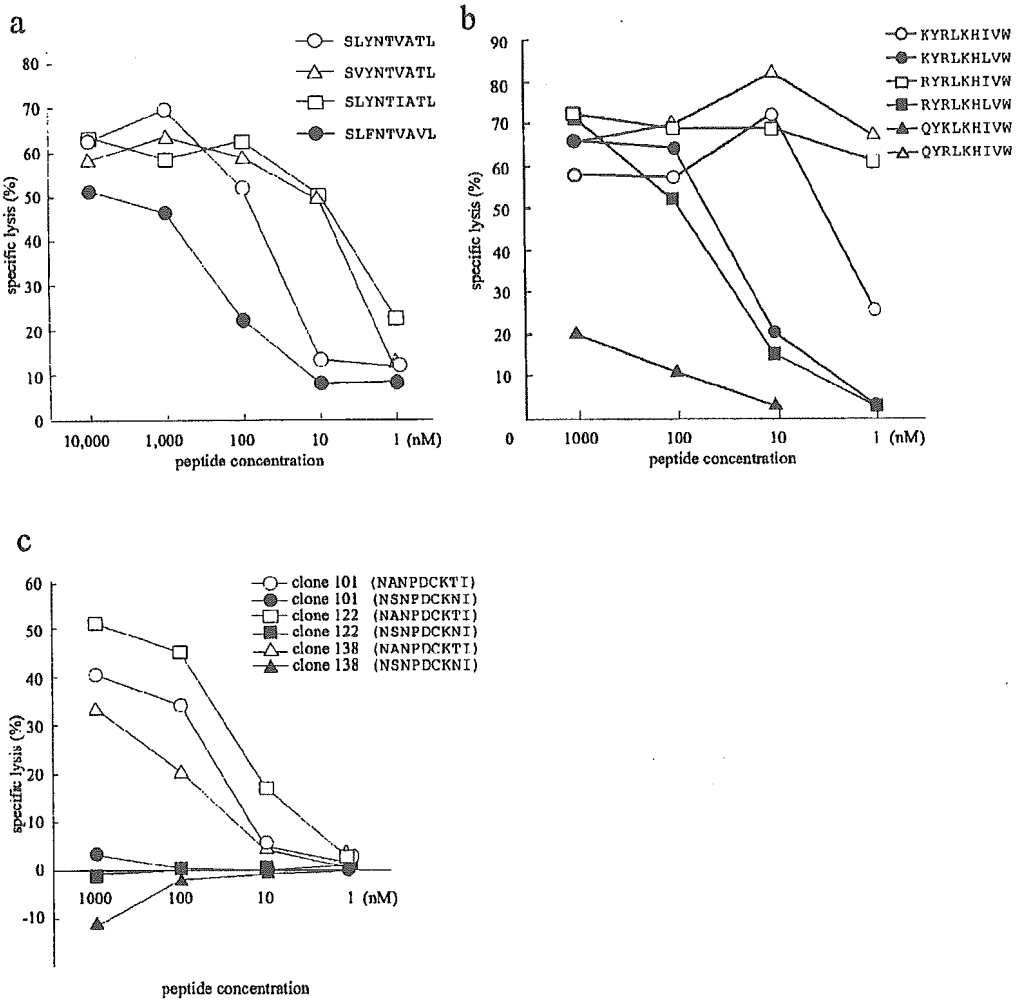

FIG. 4. Peptide titration assays. (a) Specific lysis of A*0201-matched B-LCLs pulsed with A*0201 variant peptides by A*0201-restricted CTLs at an E:T ratio of 20:1. (b) Specific lysis of A24-matched B-LCLs pulsed with 3R and its variant peptides by A24-restricted 3R mutant reactive CTLs at an E:T ratio of 20:1. (c) Specific lysis of B51-matched B-LCLs pulsed with B51 variant peptides by B51-restricted CTL clones at an E:T ratio of 2:1. The same effector and target cells were used as for Fig. 3. The percent lysis of the blank control has been subtracted.

wild-type epitope sequence restored CTL recognition of the escape variants, whereas replacement of the wild-type epitope with the two variant epitopes resulted in no CTL recognition of HXB2 Gag (Fig. 5a). The levels and patterns of Gag protein expression in target cells were analyzed by Western blot experiments (Fig. 5b). The expression levels of p55 Gag precursor and p24 CA did not significantly differ between the mutants and the wild type. The p17 MA band was not clear in HXB2-2V, IMS7-11-wild, and IMS4-24-wild, but the appearance of this band did not correlate with CTL killing. These results indicate that amino acid substitutions within the A*0201-restricted epitope region, rather than those in the flanking regions, have caused the inhibition of CTL recognition in our endogenous expression system.

To further investigate the effect of amino acid substitutions within the A24-restricted epitope on antigen processing and presentation, we introduced various point mutations into the wild-type HXB2 vector, pCTLpac, and tested them for the recognition by A24-restricted 3R mutant-reactive CTL lines. The A24-restricted 3R mutant-specific CTLs did not cross-recognize the wild-type peptide and the wild-type HXB2 vector but did recognize HXB2 with a 3R mutation (HXB2-1R). The substitution of Lys (K) with Arg (R) at position 28 (HXB2-1R3R) did not affect the A24-restricted 3R mutant-specific CTL recognition, but a Lys (K)-to-Gln (Q) substitution at position 28 (HXB2-1Q3R) or an Ile (I)-to-Leu (L) substitution at position 34 (HXB2-3R7L) resulted in the escape from CTL killing (Fig. 5c).

Replication kinetics of HIV-1 mutant viruses. We analyzed the replication kinetics of recombinant viruses carrying mutations that have affected the epitope processing and presentation by infecting H9 or Jurkat cells. All mutants were found to replicate to equivalent levels, suggesting that these mutations do not have a significant influence on HIV-1 replication (Fig. 6).

DISCUSSION

The present study focused on three Gag CTL epitopes restricted by three common HLA alleles in Japanese people (24).

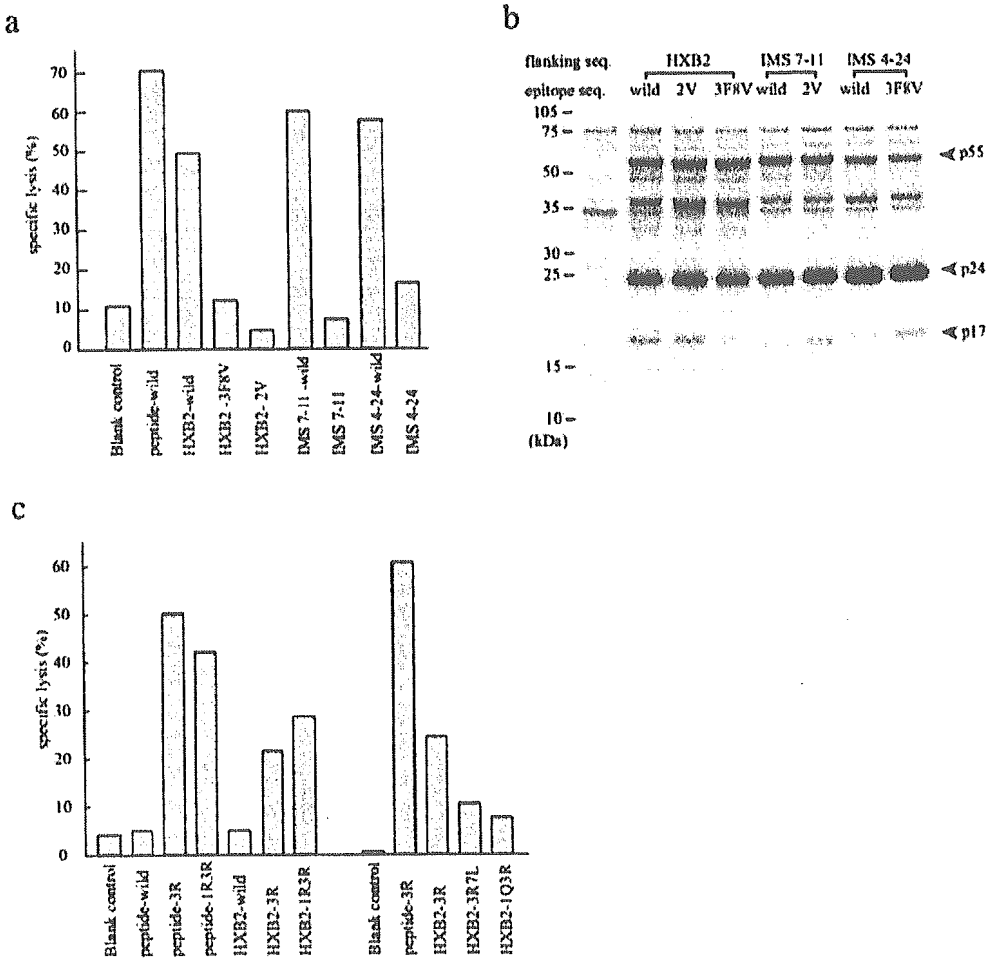

FIG. 5. (a) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) that endogenously express chimeric *gag* clones bearing the variant CTL epitopes SLFNTVAVL and SVYNTVATL in the frame of HXB2 *gag* (HXB2-3F8V and HXB2-2V, respectively) or bearing the wild-type epitope in the frame of IMS7-11 and IMS4-24 Gag (IMS7-11-wild and IMS4-24-wild, respectively). A*0201-restricted SLYNTVATL CTL lines were induced from the same donor as for Fig. 3. Specific lysis of target cells expressing HXB2, IMS7-11, or IMS4-24 *gag* clones and being pulsed with the A*0201 wild-type peptide (10 μM) is shown in parallel. The E:T ratio was 20:1. (b) Levels and patterns of HIV-1 protein expression in target cells used in the experiments described for panel a. The Western blot was reacted with the serum from an HIV-1-infected individual. (c) Specific lysis of A24-matched B-LCLs (HLA A24/− and HLA-B46/52 or HLA-A24/26 and HLA-B51/52) that express *gag* clones with various point mutations. Point mutations were inserted into the A24-restricted CTL epitope region in the frame of wild-type HXB2 Gag (HXB2-wild): amino acid substitutions of Lys to Arg at position 30 (HXB2-3R) with Lys to Arg at position 28 (HXB2-1R3R), Ile to Lue at position 34 (HXB2-3R7L), or Lys to Gln at position 28 (HXB2-1Q3R). Peptide target cells were pulsed with either the KYRLKHIVW (3R) or the RYRLKHIVW (1R3R) mutant peptide at 10 μM. The effector cells were A24-restricted 3R mutant-specific CTL lines from the same donor as in the Fig. 3e experiment. The E:T ratio was 20:1.

The Gag protein is most commonly targeted by CTL-inducing HIV/AIDS vaccines (15). In our endogenous expression system, three A*0201-restricted epitope variants and one B*5101-restricted epitope variant escaped from the wild-type CTL recognition, and four A24-restricted epitope variants escaped from the A24-restricted 3R mutant-reactive CTL recognition. Intriguingly, two A*0201-restricted variants and three A24-restricted variants escaped from CTL killing when the *gag* clones were expressed endogenously in the target cells by the HIV-1 vector, despite the fact that the synthetic variant peptides were well recognized by the CTLs when loaded onto the MHC class I molecule exogenously. The peptide titration experiments have revealed that the strength of these variant peptides' recognition was almost equivalent to that of the A*0201-restricted wild-type peptide or the A24-restricted 3R mutant peptide. The results were not likely due to differences in the pattern of Gag protein expression, as shown in the Western blot experiments. All target cells were confirmed to express a sufficient level of Gag protein by p24 antigen production. Therefore, we believe that the escape mechanism of these variants resides in the antigen processing and presentation, as has been observed in a mouse model with murine leukemia virus infection (19). The observation of such phenomenon in two epitopes restricted by different alleles implies that this finding is not unique to a particular epitope-MHC pair.

Since all variants investigated here were derived from clinical samples and those mutations did not affect the virus replication, our observations are relevant for discussing what may be going on in HIV-infected individuals. Our results indicate

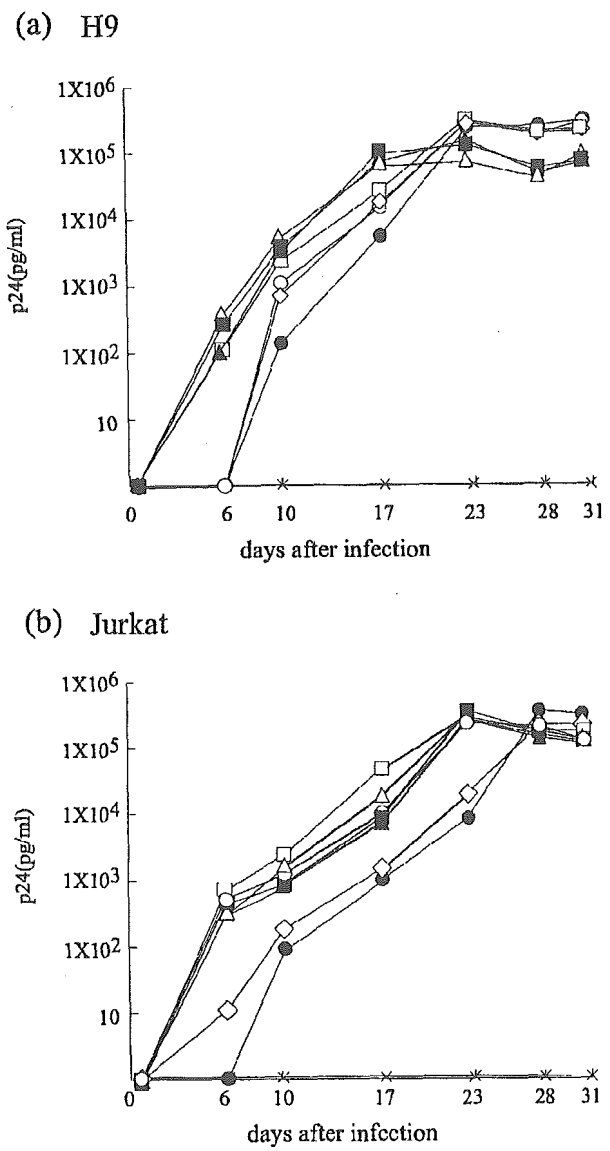

FIG. 6. Replication of HIV-1 clones with mutations that impaired the processing and presentation of A*0201 or A24 CTL epitopes in H9 (a) and Jurkat (b) cells. The kinetics of each recombinant virus replication were monitored as the production of p24 antigen by p24 ELISA. Symbols: ○, wild-type; □, A24-3R; ■, A24-K26S+3R; △, A24-3R7L; ▲, A24-1Q3R; ●, A*0201-3F8V; ◇, A*0201-2V; ×, mock.

that the impaired antigen processing and presentation often occurs in HIV-1 field isolates and thus is one of the major mechanisms that enable HIV-1 to escape from the CTL recognition. To understand further the significance of this escape mechanism, it is important to evaluate an accumulation of such escape variants in infected hosts in a longitudinal study or at a population level. A previous report using a vaccinia virus expression system did not reveal that any mutations in the A*0201-restricted p17 epitope of HIV-1 and its flanking region altered the processing and presentation of its variant epitope (4). However, that study did not investigate A*0201-restricted 2V and 3F8V variants, which we found affected epitope processing and presentation.

Experiments with chimeric genes, as well as point mutations, showed that escapes from epitope processing and presentation were mostly attributable to mutations within the epitope regions rather than its flanking regions. In the present study, we demonstrated that point mutations of Lys (L) to Gln (Q) at position 28 and of Ile to Leu at position 34 drastically impaired the processing and presentation of the A24-restricted CTL epitope. Moreover, the experiment with HXB2 clone carrying IMS 7-11 variant of A*0201-restricted CTL epitope indicates that a substitution of Leu (L) to Val (V) at position 78 was responsible for the impaired processing and presentation of the epitope. These mutations in the epitope region may have induced a proteasome cleavage site within the epitope (19). On the other hand, we observed that the variations in the 15 amino acids up- and downstream of the epitope did not affect CTL recognition. An exception was a Lys (L)-to-Ser (S) substitution (−2S) at position 26, which is only two amino acids adjacent to the N terminus of the A24-restricted epitope. However, this −2S substitution did not void the A24-restricted 3R mutant-reactive CTL recognition completely. One possible explanation is that the −2S substitution shifted the optimal proteasome cleavage site, resulting in the generation of a larger peptide, which has a lower affinity to the MHC class I molecule.

We have first attempted to investigate the antigen processing and presentation by the conventional recombinant vaccinia virus method for all variants before we established this VSV-G-pseudotyped HIV-1 vector method. Soon, we realized that preparing recombinant vaccinia viruses was much more laborious and time-consuming. Early experiments of comparing two methods by using the first available recombinant vacinia viruses concluded that the HIV-1 vector method demonstrated CTL killing better than did the recombinant vaccinia virus method (Fig. 3b). In the recombinant vaccinia virus expression system, the massive production of vaccinia virus proteins inevitably takes place, along with the expression of an HIV-1 gene and sometimes causes a high background lysis. The expression manner and the production ratio to non-HIV proteins may also influence antigen processing and presentation (27, 34). Thus, we thought that the antigen processing and presentation in the HIV-1 vector expression system is more physiological than the recombinant vaccinia virus expression system and that continuing vaccinia virus experiments would not be significantly beneficial to address the issue of antigen processing and presentation. Nevertheless, there remains a concern that there might be a potential difference in the antigen processing and presentation between immortalized B cells that were used here and primary $CD4^+$ T cells (32, 33). Perhaps it is important to reevaluate the interaction of CTLs and these variants in experiments with variant HIV-1-infected T cells. Our HIV-1 vector carries neither the *nef* gene nor the *vpu* gene, which significantly affect antigen presentation by downregulating MHC class I cell surface expression (5, 13). From this point of view, one might expect that more variants would escape from the CTL recognition in the actual HIV-1 infection than what is shown in our experiments. However, we think that our system is suited to identify a specific association between a certain mutation and the escape from antigen processing and presentation. To prove the existence of this mode of escape mecha-

nism, we may need a new system that can directly detect a trace of specific epitopes that are eluted from MHC class I molecules of HIV-1 antigen-producing cells.

Although the structure analysis of MHC class I molecules and its binding motif has facilitated the prediction of CTL epitopes from the primary amino acid sequence data of HIV-1 (6, 11, 26), it remains difficult to envisage the efficiency of epitope processing and presentation. Enormous diversity realized in HIV-1 field isolates causes a further complexity (7). Our data emphasize the importance of testing HIV-1 variants in an endogenous expression system. Detailed analysis of epitope processing and presentation among HIV-1 field isolates, particularly of non-B subtypes circulating in the vaccine trial fields, is essential, since such information allows us to forecast which virus may elude the immunity elicited by vaccines, thus providing a clue for a rational design for effective HIV/AIDS vaccines.

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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

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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.

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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	F	K	D	T	K	I	V	V	Q	M	I	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.

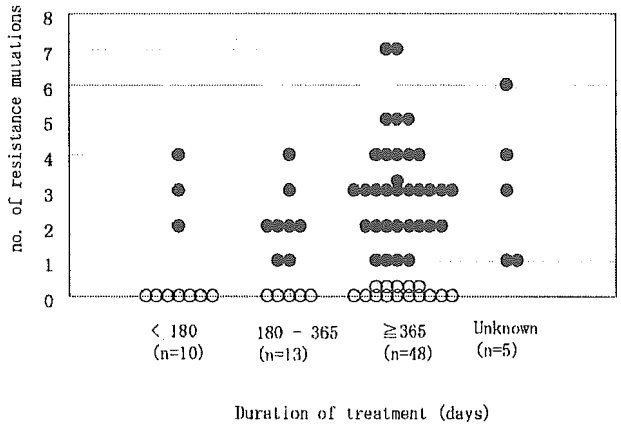

FIGURE 2. Prevalence of AZT, ddI, or ddC resistance mutations in relation to the duration of the dual therapy. Open circles indicate samples without resistance mutations.

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.

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