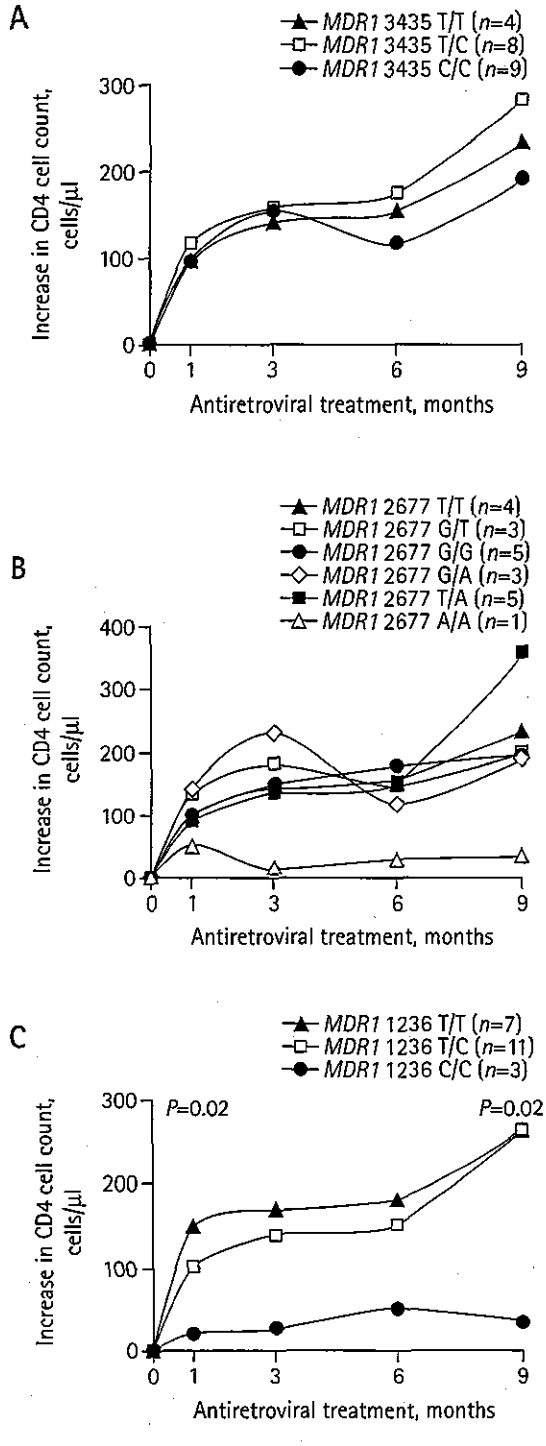
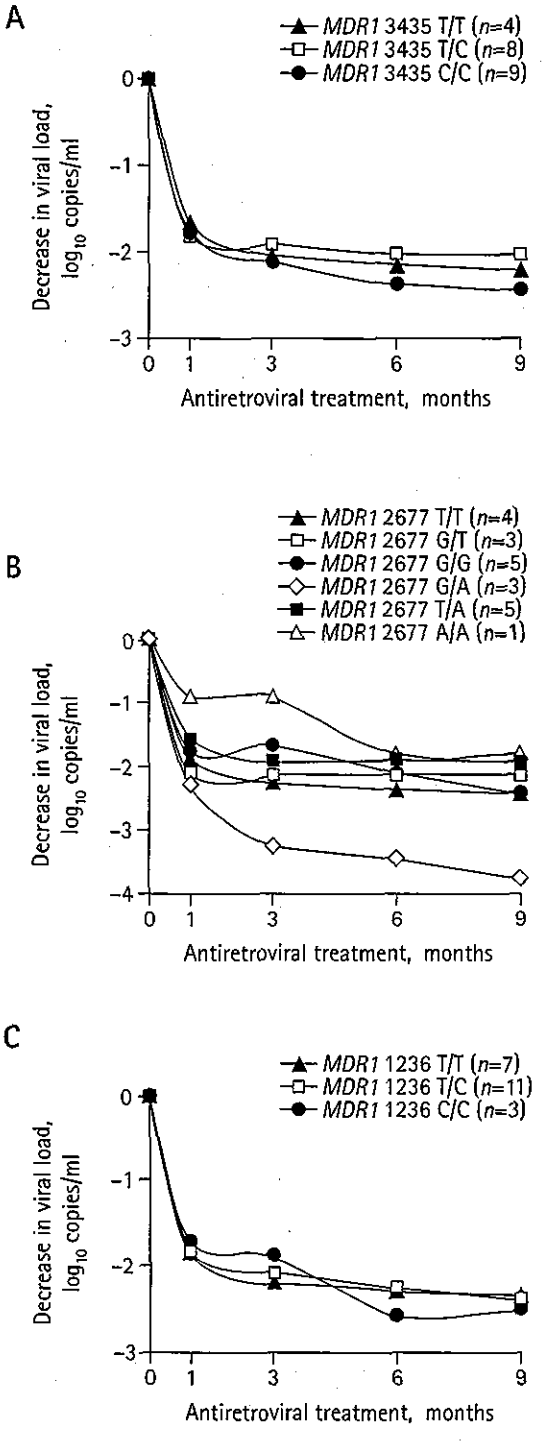
Figure 5. Increase in CD4 cell count among patients with the various genotypes of *MDR1* during antiretroviral treatment



We assessed increase in CD4 cell counts among 21 patients. Every subject had CD4 cell counts and viral loads at months 0, 1, 3, 6 and 9. (A) *MDR1* 3435: T/T (▲); C/C (●); T/C (□). (B) *MDR1* 2677: T/T (▲); G/G (●); G/T (□); G/A (◇); T/A (■); A/A (△). (C) *MDR1* 1236: T/T (▲); C/C (●); T/C (□). The vertical axis shows the increase in CD4 cell count during treatment. *P* values were calculated by the Mann–Whitney U-test.

Figure 6. Suppression of viraemia among patients with various genotypes of *MDR1* after antiretroviral treatment



We assessed suppression of viraemia among the same 21 patients as described in the legend to Figure 5. (A) *MDR1* 3435: T/T (▲); C/C (●); T/C (□). (B) *MDR1* 2677: T/T (▲); G/G (●); G/T (□); G/A (◇); T/A (■); A/A (△). (C) *MDR1* 1236: T/T (▲); C/C (●); T/C (□). The vertical axis shows decrease in viral load. Values are shown as $\log_{10}$ copies/ml plasma.

receiving PIs, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 SNPs (Figures 5A and 6A). Furthermore, we found that patients with the *MDR1* 1236 T/T genotype showed a greater increase in the CD4 cell counts during HAART therapy with PI at months 1 and 9 than patients with the *MDR1* 1236 C/C genotype (Figure 5C). The contribution of genetic variations in the *MDR1* gene to the patients' clinical characteristics, if any, seems very complicated and thus is difficult to evaluate in a straightforward manner.

As the steady-state intracellular concentration of NFV was about 250 times higher than that in the medium (10 μM), the uptake of NFV seems active rather than passive. However, these *in vitro* data depart from what has been observed in *in vivo* measurements of NFV in patients [19,20], presumably due to the presence of alpha(1)-acid glycoprotein to which NFV binds in plasma [21]. Furthermore, this discrepancy may also be due to the differential distribution of NFV among tissues rather than in free artificial medium. Therefore, our *in vitro* data should be considered as such, that is, *in vivo* lymphocytes may be unlikely to have this high intracellular to extracellular concentration ratio (250:1).

We observed an association of slower efflux of NFV *in vitro* with the T/T genotype at *MDR1* 3435. In fact, P-gp has been found to export PIs from lymphocytes and reduce their anti-HIV activity *in vitro*, and its low activity has been found to be associated with the T/T genotype at *MDR1* 3435 [13]. As the SNP at *MDR1* 3435 is a silent mutation, one possible explanation for this association is that the T/T genotype at *MDR1* 3435 renders *MDR1* mRNA unstable in the cell. Another possible explanation for the association is that *MDR1* 3435 SNP is in linkage disequilibrium with the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 2677 (exon 21), the latter of which is a substitution mutation. This amino acid substitution from the *MDR1* 2677 SNP may be responsible for the observed difference (Figure 4) [11]. Another possible explanation is that *MDR1* 3435 SNPs are in linkage disequilibrium with a polymorphism(s) elsewhere in the genome that modifies *MDR1* expression or function [3,12].

Although an *in vitro* study showed that the velocity of NFV efflux in patients' LCLs with the *MDR1* 3435 T/T genotype was slower than that with the C/C genotype, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 genotypes (Figures 5A and 6A). Four equally possible accounts seem to explain this discrepancy. Firstly, since the C/C genotype at *MDR1* 3435 is also correlated with higher expression of P-gp in intestinal epithelial cells that adsorb PIs, the *MDR1* 3435 C/C is likely to be associated with higher absorption of PIs and higher PI concentration in plasma [12,22]. The higher plasma levels of NFV in 3435 C/C patients in one study [12] is puzzling and as yet not fully understood. Secondly, the sample size (n=21) in this study may be too small to evaluate CD4 cell counts or viral suppression in a statistical way. Thirdly, since the enrolled patients received different treatment combinations of PIs and reverse transcription inhibitors during antiretroviral therapy, the clinical evaluation was not normalized. Finally, because LCLs – immobilized B cells – but not CD4+ T cells were used in this study, the function of P-gp in a setting of HIV-1 infection may not have been accurately tested. In contrast to the *MDR1* 3435, we observed a marginal but statistically significant association of the *MDR1* 1236 SNP with the CD4 cell count increase although this SNP is a silent mutation. To our knowledge, this clinical association of *MDR1* 1236 with statistical significance is unprecedented, although its clinical significance remains to be investigated. In conclusion, a large-scale and case-controlled study would be required to test whether SNPs of *MDR1* affect the clinical course during antiretroviral therapy with PIs and the prognosis of infected patients.

Acknowledgements

This work was partly supported by grants for AIDS research from the Ministry of Health, Labour and Welfare of Japan, Grant-in-Aid for Scientific Research (A) from the Japan Society of the Promotion of Science (JSPS) and the Japan Health Sciences Foundation. We thank Dr Yusuke Nakamura (IMSUT) for Japanese SNP information.

References

1. Chaillou S, Durant J, Garraffo R, Georgenthum E, Roptin C, Dunais B, Mondain V, Roger PM & Dellamonica P. Intracellular concentration of protease inhibitors in HIV-1-infected patients: correlation with MDR-1 gene expression and low dose of ritonavir. *HIV Clinical Trials* 2002; 3:493–501.
2. Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I & Dey S. HIV-1 protease inhibitors are substrates for the *MDR1* multidrug transporter. *Biochemistry* 1998; 37:3594–3601.
3. Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmoller J, Johne A, Cascorbi I, Gerloff T, Roots I, Eichelbaum M & Brinkmann U. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo*. *Proceedings of the National Academy of Sciences, USA* 2000; 97:3473–3478.
4. Chaudhary PM, Mechetner EB & Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood* 1992; 80:2735–2739.
5. Drach D, Zhao S, Drach J, Mahadevia R, Gattringer C, Huber H & Andreeff M. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* 1992; 80:2729–2734.

6. Sakaeda T, Nakamura T & Okumura K. MDR1 genotype-related pharmacokinetics and pharmacodynamics. *Biological & Pharmaceutical Bulletin* 2002; 25:1391–1400.
7. Yacyshyn B, Maksymowych W & Bowen-Yacyshyn MB. Differences in P-glycoprotein-170 expression and activity between Crohn's disease and ulcerative colitis. *Human Immunology* 1999; 60:677–687.
8. Bellamy WT. P-glycoproteins and multidrug resistance. *Annual Review of Pharmacology & Toxicology* 1996; 36:161–183.
9. Mickley LA, Lee JS, Weng Z, Zhan Z, Alvarez M, Wilson W, Bates SE & Fojo T. Genetic polymorphism in *MDR-1*: a tool for examining allelic expression in normal cells, unselected and drug-selected cell lines, and human tumors. *Blood* 1998; 91:1749–1756.
10. Saito S, Iida A, Sekine A, Miura Y, Ogawa C, Kawauchi S, Higuchi S & Nakamura Y. Three hundred twenty-six genetic variations in genes encoding nine members of ATP-binding cassette, subfamily B (ABCB/MDR/TAP), in the Japanese population. *Journal of Human Genetics* 2002; 47:38–50.
11. Tanabe M, Ieiri I, Nagata N, Inoue K, Ito S, Kanamori Y, Takahashi M, Kurata Y, Kigawa J, Higuchi S, Terakawa N & Otsubo K. Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance *(MDR)-1* gene. *Pharmacology & Experimental Therapeutics* 2001; 297:1137–1143.
12. Fellay J, Marzolini C, Meaden ER, Back DJ, Buclin T, Chave JP, Decosterd LA, Furrer H, Opravil M, Pantaleo G, Retelska D, Ruiz L, Schinkel AH, Vernazza P, Eap CB & Telenti A. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. *Lancet* 2002; 359:30–36.
13. Hitzl M, Drescher S, van der Kuip H, Schaffeler E, Fischer J, Schwab M, Eichelbaum M & Fromm MF. The C3435T mutation in the human *MDR1* gene is associated with altered efflux of the P-glycoprotein substrate rhodamine 123 from CD56+ natural killer cells. *Pharmacogenetics* 2001; 11:293–298.
14. Nasi M, Borghi V, Pinti M, Bellodi C, Lugli E, Maffei S, Troiano L, Richeldi L, Mussini C, Esposito R & Cossarizza A. *MDR1* C3435T genetic polymorphism does not influence the response to antiretroviral therapy in drug-naive HIV-positive patients. *AIDS* 2003; 17:1696–1698.
15. Kobayashi N, Nakamura HT, Goto M, Nakamura T, Nakamura K, Sugiura W, Iwamoto A & Kitamura Y. Polymorphisms and haplotypes of the *CD209L* gene and their association with the clinical courses of HIV-positive Japanese patients. *Japanese Journal of Infectious Diseases* 2002; 55:131–133.
16. Miller G & Lipman M. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proceedings of the National Academy of Sciences, USA* 1973; 70:190–194.
17. Taipalensuu J, Tornblom H, Lindberg G, Einarsson C, Sjoqvist F, Melhus H, Garberg P, Sjostrom B, Lundgren B & Artursson P. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. *Pharmacology & Experimental Therapeutics* 2001; 299:164–170.
18. Yamada H, Kotaki H, Nakamura T & Iwamoto A. Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir and nelfinavir in human plasma by high-performance liquid chromatography. *Journal of Chromatography. B, Biomedical Sciences & Applications* 2001; 755:85–89.
19. Hennessy M, Clarke S, Spiers JP, Kelleher D, Mulcahy F, Hoggard P, Back D & Barry M. Intracellular accumulation of nelfinavir and its relationship to P-glycoprotein expression and function in HIV-infected patients. *Antiviral Therapy* 2004; 9:115–122.
20. Ford J, Cornforth D, Hoggard PG, Cuthbertson Z, Meaden ER, Williams I, Johnson M, Daniels E, Hsyu P, Back DJ & Khoo SH. Intracellular and plasma pharmacokinetics of nelfinavir and M8 in HIV-infected patients: relationship with P-glycoprotein expression. *Antiviral Therapy* 2004; 9:77–84.
21. Schon A, del Mar Ingaramo M & Freire E. The binding of HIV-1 protease inhibitors to human serum proteins. *Biophysical Chemistry* 2003; 105:221–230.
22. Kim RB, Leake BF, Choo EF, Dresser GK, Kubba SV, Schwarz UI, Taylor A, Xie HG, McKinsey J, Zhou S, Lan LB, Schuetz JD, Schuetz EG & Wilkinson GR. Identification of functionally variant *MDR1* alleles among European Americans and African Americans. *Clinical Pharmacology & Therapeutics* 2001; 70:189–199.

Received 26 March 2004, accepted 11 August 2004

JOURNAL OF VIROLOGY, Feb. 2004, p. 1324–1332 Vol. 78, No. 3
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.3.1324–1332.2004

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

Yoshiyuki Yokomaku,[1] Hideka Miura,[1] Hiroko Tomiyama,[2] Ai Kawana-Tachikawa,[3] Masafumi Takiguchi,[2] Asato Kojima,[4] Yoshiyuki Nagai,[1]† Aikichi Iwamoto,[3] Zene Matsuda,[1] and Koya Ariyoshi[1]*

AIDS Research Center[1] and Department of Pathology,[4] National Institute of Infectious Diseases, and Department of Infectious Diseases, Institute of Medical Science, University of Tokyo,[3] Tokyo, and Center for AIDS Research, Kumamoto University, Kumamoto,[2] Japan

Received 21 April 2003/Accepted 7 October 2003

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

* Corresponding author. Mailing address: AIDS Research Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo, 208-0011 Japan. Phone: 81-42-561-0771. Fax: 81-42-561-7746. E-mail: ariyoshi@nih.go.jp

†Present address: Toyama Institute of Health, Toyama, 939-0363 Japan.

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

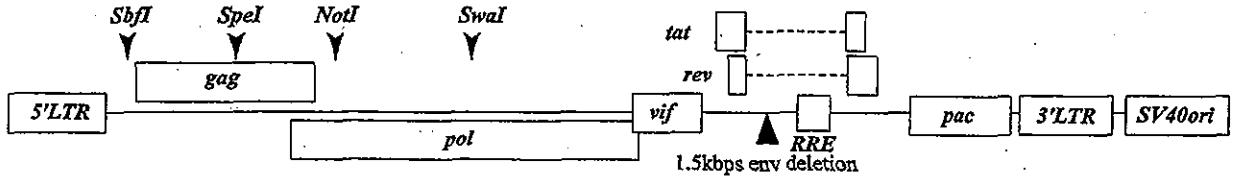


FIG. 1. Structure of pCTLpac. A 1.5-kbp portion of *env* was deleted (▲). Puromycin *N*-acetyltransferase gene (*pac*) was inserted in the *nef* region. The locations of restriction enzyme sites are indicated (▼). RRE, Rev responsive element.

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

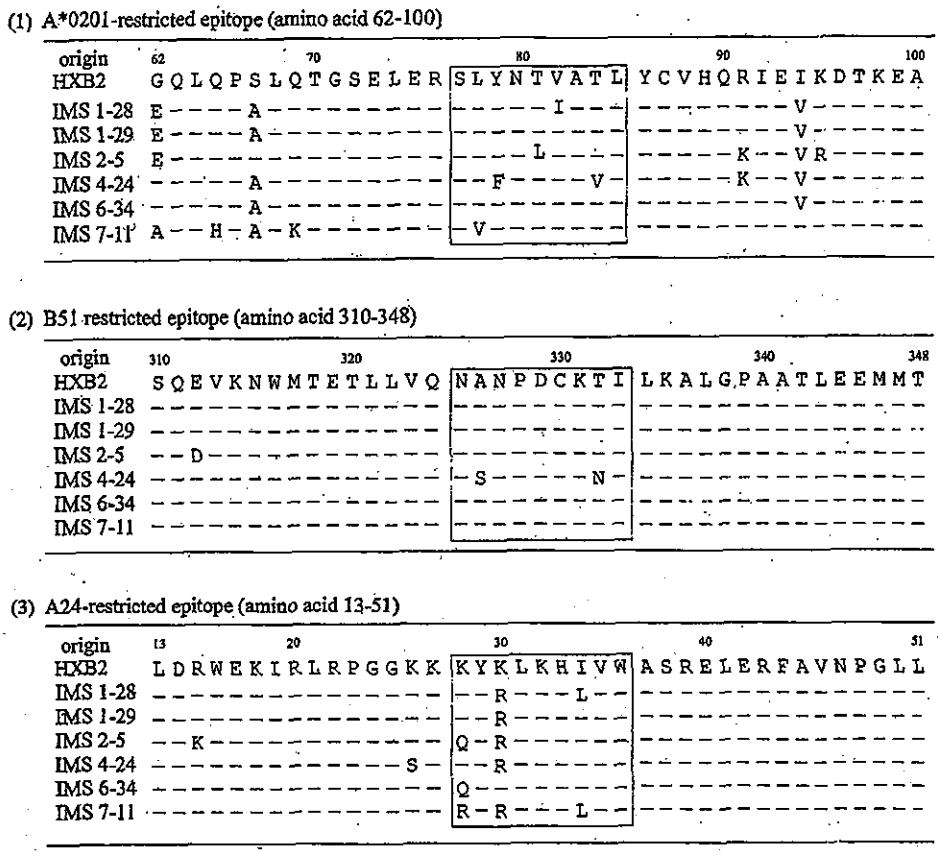


FIG. 2. Sequence variation in three CTL epitopes and their flanking regions. The amino acid sequences of six *gag* clones are shown. The reference sequence is derived from HXB2, and the differences are indicated. The numbering is done according to the HIV sequence database, Los Alamos National Laboratory,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

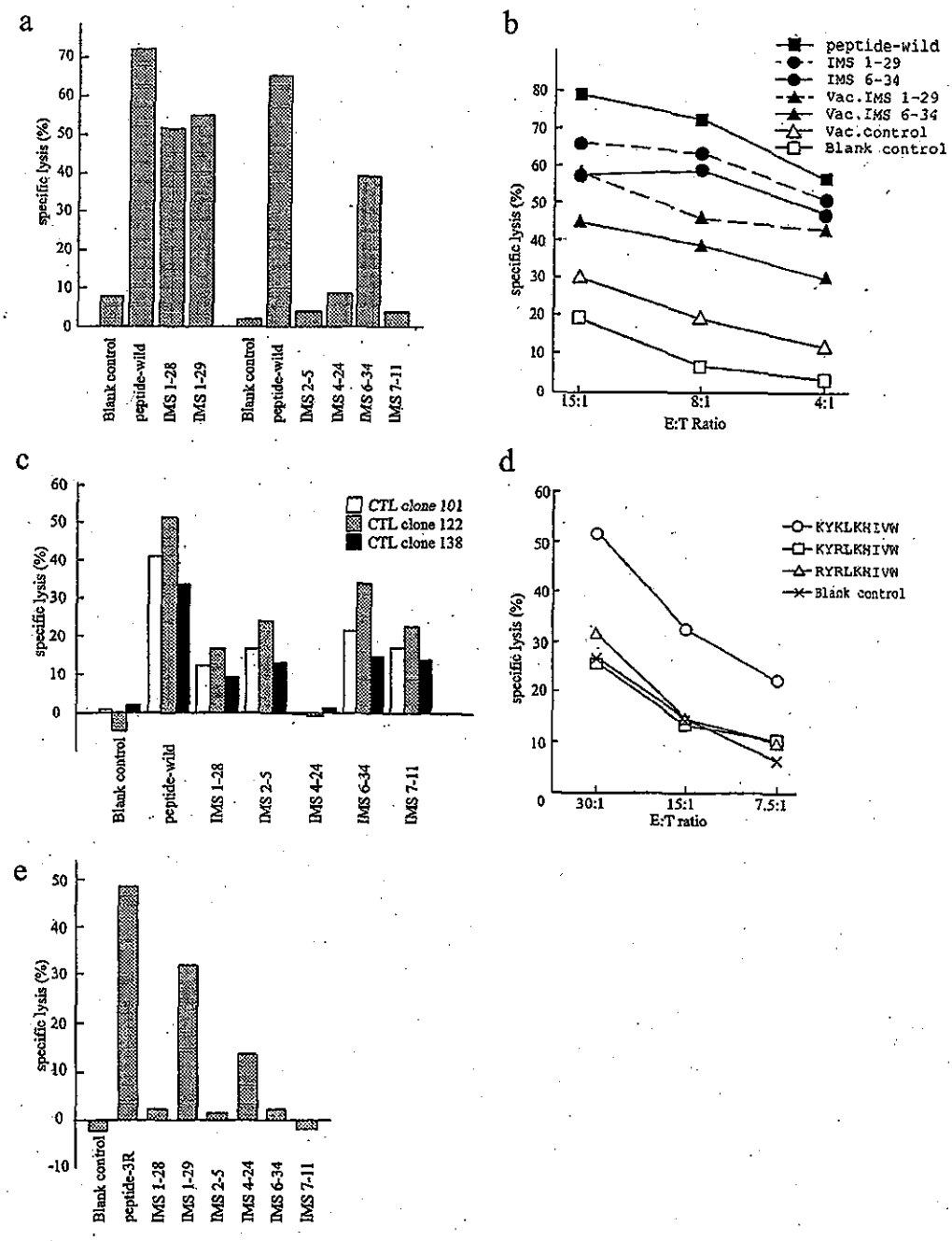


FIG. 3. (a) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) producing Gag proteins of clinical isolates. Peptide target cells were pulsed with the A*0201 wild-type peptide, SLYNTVATL (10 μM). A*0201-restricted SLYNTVATL -specific CTL lines were induced from a single donor (IMS1). 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.

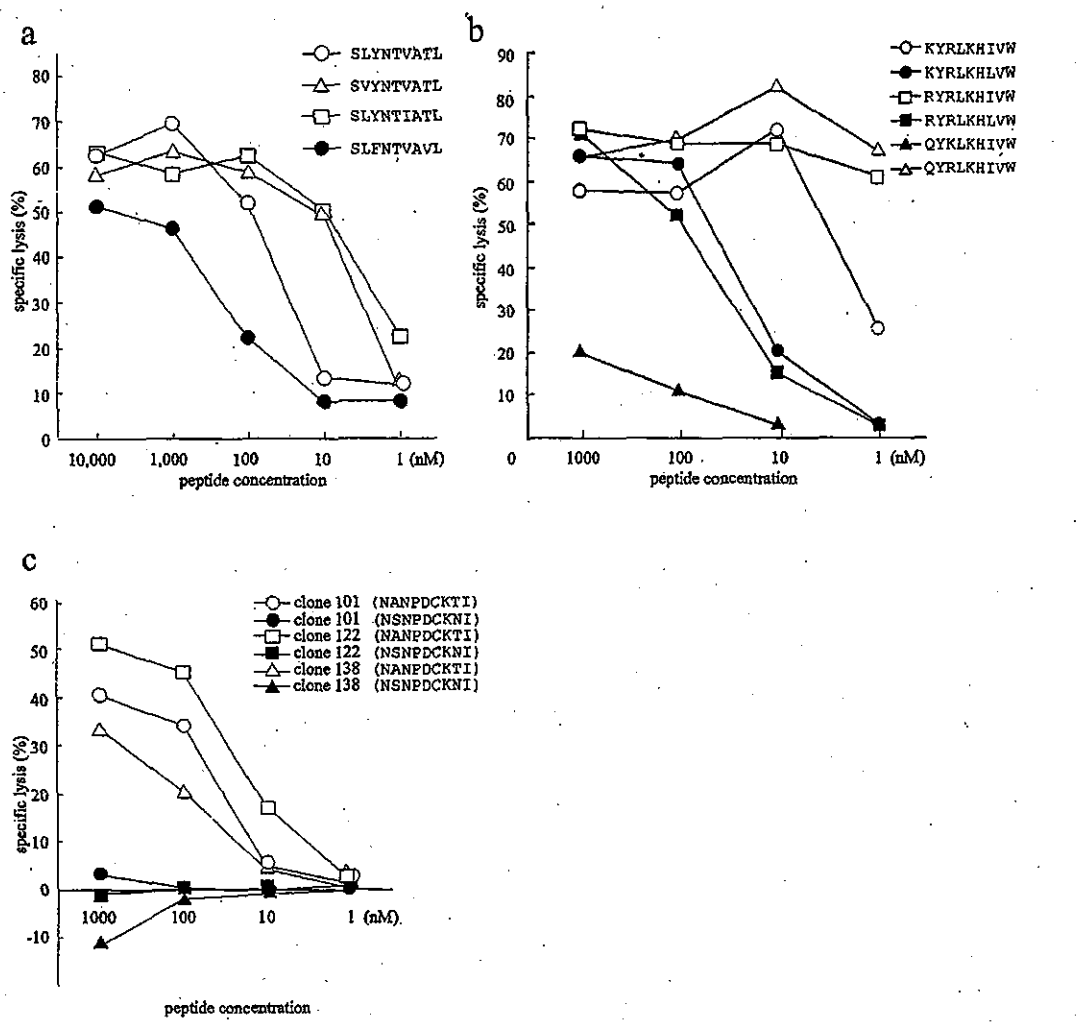


FIG. 4. Peptide titration assays. (a) Specific lysis of A*0201-matched B-LCLs pulsed with A*0201 variant peptides by A*0201-restricted CTLs at an E:T ratio of 20:1. (b) Specific lysis of A24-matched B-LCLs pulsed with 3R and its variant peptides by A24-restricted 3R mutant reactive CTLs at an E:T ratio 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).

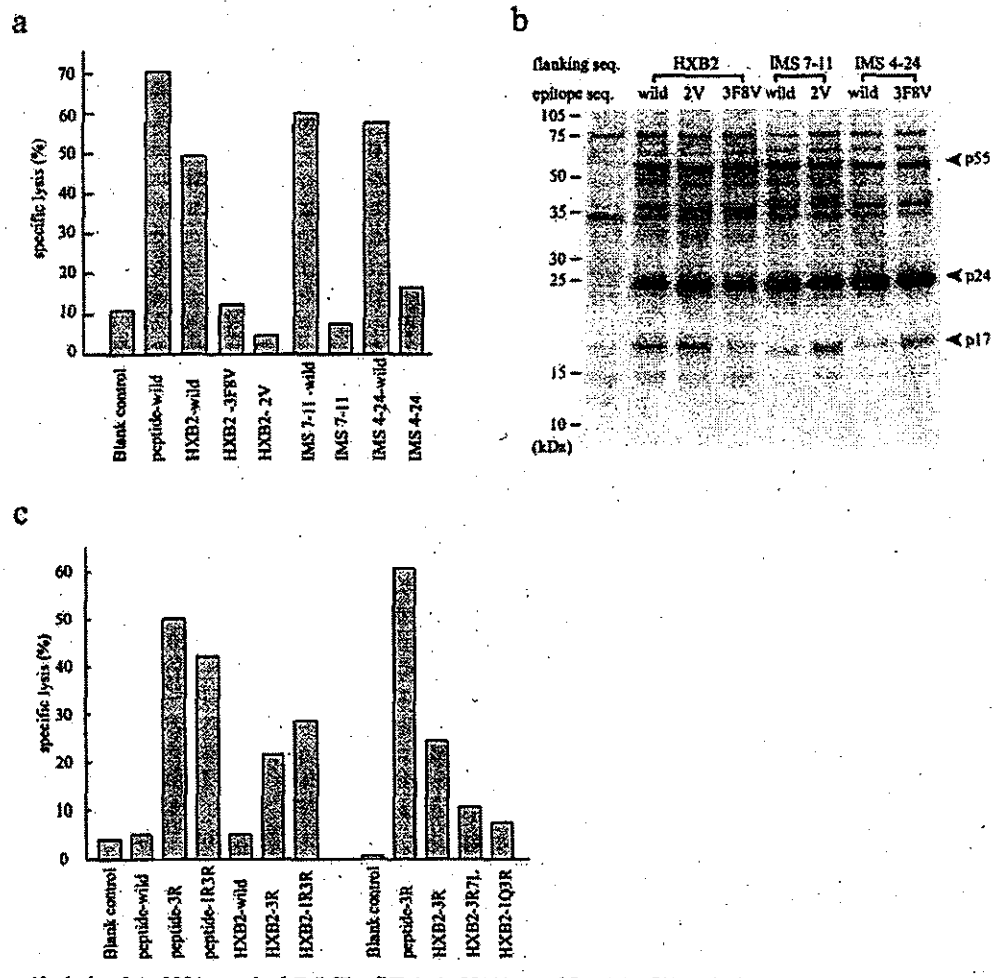


FIG. 5. (a) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) that endogenously express chimeric *gag* clones bearing the variant CTL epitopes SLFNTVAVL and SVYNTVATL in the frame of HXB2 *gag* (HXB2-3F8V and HXB2-2V, respectively) or bearing the wild-type epitope in the frame of 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

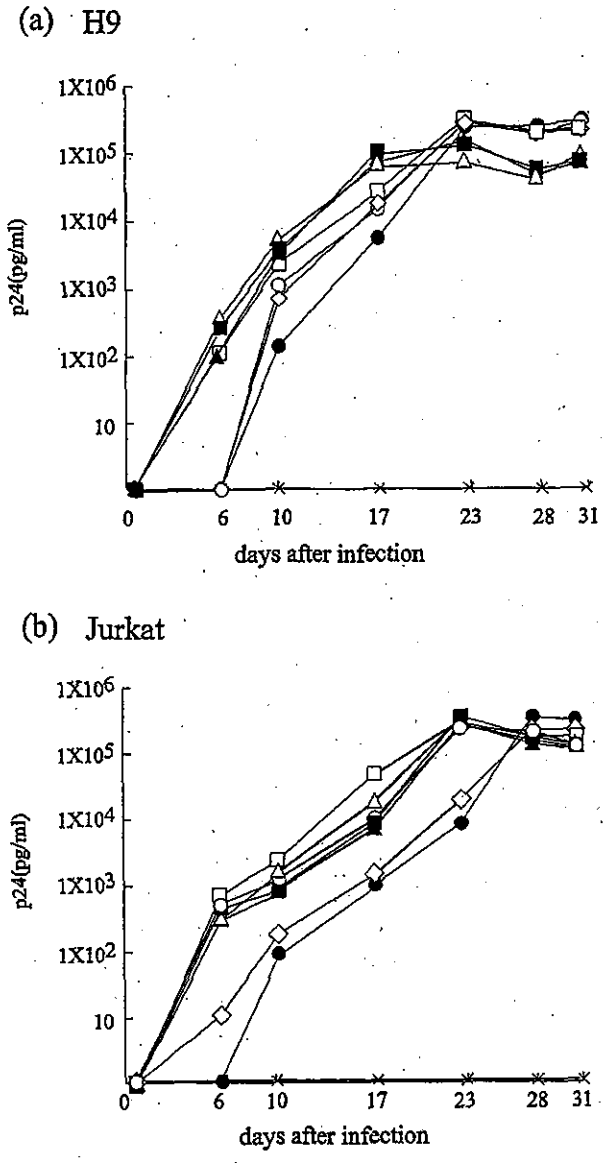


FIG. 6. Replication of HIV-1 clones with mutations that impaired the processing and presentation of A*0201 or A24 CTL epitopes in H9 (a) and Jurkat (b) cells. The kinetics of each recombinant virus replication were monitored as the production of p24 antigen by p24 ELISA. Symbols: ○, wild-type; □, A24-3R;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

ACKNOWLEDGMENTS

We thank the donors in this study for their participation, Sachiko Tateishi for assistance, and Kunito Yoshiike for critical reading of the manuscript.

This study was supported by the Japanese Ministry of Health, Labor, and Welfare and the Japan Health Science Foundation.

REFERENCES

1. **Aoki, N., T. Shioda, H. Satoh, and H. Shibuta.** 1991. Syncytium formation of human and non-human cells by recombinant vaccinia viruses carrying the HIV *env* gene and human CD4 gene. AIDS 5:871–875.
2. **Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin.** 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 415:335–339.
3. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. 3:205–211.
4. **Brander, C., O. O. Yang, N. G. Jones, Y. Lee, P. Goulder, R. P. Johnson, A. Trocha, D. Colbert, C. Hay, S. Buchbinder, C. C. Bergmann, H. J. Zweerink, S. Wolinsky, W. A. Blattner, S. A. Kalams, and B. D. Walker.** 1999. Efficient processing of the immunodominant, HLA-A*0201-restricted human immunodeficiency virus type 1 cytotoxic-T-lymphocyte epitope despite multiple variations in the epitope flanking sequences. J. Virol. 73:10191–10198.
5. **Collins, K. L., B. K. Chen, S. A. Kalams, B. D. Walker, and D. Baltimore.** 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature 391:397–401.
6. **Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, and H. G. Rammensee.** 1991. *Allele-specific motifs revealed by sequencing* of self-peptides eluted from MHC molecules. Nature 351:290–296.
7. **Gaschen, B., J. Taylor, K. Yusim, B. Foley, F. Gao, D. Lang, V. Novitsky, B. Haynes, B. H. Hahn, T. Bhattacharya, and B. Korber.** 2002. Diversity considerations in HIV-1 vaccine selection. Science 296:2354–2360.
8. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. 3:212–217.
9. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123–126.
10. **Hoshikawa, N., A. Kojima, A. Yasuda, E. Takayashiki, S. Masuko, J. Chiba, T. Sata, and T. Kurata.** 1991. Role of the gag and pol genes of human immunodeficiency virus in the morphogenesis and maturation of retrovirus-like particles expressed by recombinant vaccinia virus: an ultrastructural study. J. Gen. Virol. 72:2509–2517.
11. **Ikeda-Moore, Y., H. Tomiyama, M. Ibe, S. Oka, K. Miwa, Y. Kaneko, and M. Takiguchi.** 1998. *Identification of a novel* HLA-A24-restricted cytotoxic T-lymphocyte epitope derived from HIV-1 Gag protein. AIDS 12:2073–2074.
12. **Kawana, A., H. Tomiyama, M. Takiguchi, T. Shioda, T. Nakamura, and A. Iwamoto.** 1999. Accumulation of specific amino acid substitutions in HLA-B35-restricted human immunodeficiency virus type 1 cytotoxic T lymphocyte epitopes. AIDS Res. Hum. Retrovir. 15:1099–1107.
13. **Kerkau, T., I. Bacik, J. R. Bennink, J. W. Yewdell, T. Hunig, A. Schimpl, and U. Schubert.** 1997. The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. J. Exp. Med. 185:1295–1305.
14. **Koenig, S., A. J. Conley, Y. A. Brewah, G. M. Jones, S, Leath, L. J. Boots, V. Davey, G. Pantaleo, J. F. Demarest, C. Carter, et al.** 1995. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. Nat. Med. 1:330–336.
15. **McMichael, A. J., and T. Hanke.** 2003. HIV vaccines 1983–2003. Nat. Med. 9:874–880.
16. **McMichael, A. J., and S. L. Rowland-Jones.** 2001. Cellular immune responses to HIV. Nature 410:980–987.
17. **Momburg, F., and G. J. Hammerling.** 1998. Generation and TAP-mediated *transport of peptides for major histocompatibility complex class I molecules.* Adv. Immunol. 68:191–256.
18. **Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal.** 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science 296:1439–1443.
19. **Ossendorp, F., M. Eggers, A. Neisig, T. Ruppert, M. Groettrup, A. Sijts, E. Mengede, P. M. Kloetzel, J. Neefjes, U. Koszinowski, and C. Melief.** 1996. A single residue exchange within a viral CTL epitope alters proteasome-mediated degradation resulting in lack of antigen presentation. Immunity 5:115–124.
20. **Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, et al.** 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T-cell recognition. Nature 354:453–459.
21. **Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham, and R. E. Phillips.** 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc. Natl. Acad. Sci. USA 94:1890–1895.
22. **Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and F. Wong-Staal.** 1987. *Complete nucleotide sequences of functional* clones of the AIDS virus. AIDS Res. Hum. Retrovir. 3:57–69.
23. **Rock, K. L., and A. L. Goldberg.** 1999. Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu. Rev. Immunol. 17: 739–779.
24. **Saito, S., S. Ota, E. Yamada, H. Inoko, and M. Ota.** 2000. Allele frequencyes and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population. Tissue Antigens 56:522–529.
25. **Sugiura, W., Z. Matsuda, Y. Yokomaku, K. Hertogs, B. Larder, T. Oishi, A. Okano, T. Shiino, M. Tatsumi, M. Matsuda, H. Abumi, N. Takata, S. Shirahata, K. Yamada, H. Yoshikura, and Y. Nagai.** 2002. Interference between D30N and L90M in selection and development of protease inhibitor-resistant human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 46:708–715.
26. **Tomiyama, H., T. Sakaguchi, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, and M. Takiguchi.** 1999. Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101 molecules. Hum. Immunol. 60:177–186.
27. **Tsomides, T. J., A. Aldovini, R. P. Johnson, B. D. Walker, R. A. Young, and H. N. Eisen.** 1994. Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. J. Exp. Med. 180:1283–1293.
28. **Tsomides, T. J., B. D. Walker, and H. N. Eisen.** 1991. An optimal viral peptide recognized by $CD8^+$ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. Proc. Natl. Acad. Sci. USA 88:11276–11280.
29. **Van Baalen, C. A., M. Schutten, R. C. Huisman, P. H. Boers, R. A. Gruters, and A. D. Osterhaus.** 1998. Kinetics of antiviral activity by human immunodeficiency virus type 1-specific cytotoxic T lymphocytes (CTL) and rapid selection of CTL escape virus in vitro. J. Virol. 72:6851–6857.
30. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, et al.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373: 117–122.
31. **Wei, X., J. M. Decker, S. Wang, H Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature 422:307–312.
32. **Yang, O. O., S. A. Kalams, M. Rosenzweig, A. Trocha, N. Jones, M. Koziel, B. D. Walker, and R. P. Johnson.** 1996. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. J. Virol. 70:5799–5806.
33. **Yang, O. O., S. A. Kalams, A. Trocha, H. Cao, A. Luster, R. P. Johnson, and B. D. Walker.** 1997. Suppression of human immunodeficiency virus type 1 replication by $CD8^+$ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. J. Virol. 71:3120–3128.
34. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. *Immunol. 17:51–88.*

Available online at www.sciencedirect.com
SCIENCE DIRECT
Microbes and Infection 6 (2004) 799–805
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Functional analysis of HIV-1 *vif* genes derived from Japanese long-term nonprogressors and progressors for AIDS

Akiko Sakurai [a], Abhay Jere [a], Akiko Yoshida [a], Takeshi Yamada [b], Aikichi Iwamoto [c], Akio Adachi [a], Mikako Fujita [a,*]

[a] *Department of Virology, The University of Tokushima Graduate School of Medicine, 3-18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan*
[b] *Department of Microbiology, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan*
[c] *Department of Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan*

Received 15 March 2004; accepted 8 April 2004

Available online 07 June 2004

Abstract

We analyzed the function of human immunodeficiency virus type 1 (HIV-1) *vif* gene from Japanese long-term nonprogressors (LTNPRs) and progressors (PRs) for acquired immunodeficiency syndrome (AIDS). We constructed a basic HIV-1 infectious clone, which facilitated the incorporation and evaluation of *vif* from infected individuals. Proviral reporter clones carrying *vif* from six Japanese LTNPRs and seven PRs were then generated and their in vitro growth kinetics were analyzed. The *vif* clones, which could confer infectivity on reporter viruses, were considered active, and the ratio of the active clones to the number of clones examined per individual was determined. For the majority of LTNPRs, there was no correlation between presence or absence of functional *vif* with long-term nonprogression for AIDS. There was one exception in which all the clones examined had inactive *vif*, suggesting a probable association of inactive *vif* with the nonprogression. All PRs with high viral load had a high ratio of active *vif* clones. Our results suggest that the presence of functional *vif* would influence HIV-1 infectivity and disease progression in infected individuals.

Keywords: HIV-1; AIDS; *Vif*; Long-term nonprogressors

1. Introduction

The time between developing acquired immunodeficiency syndrome (AIDS) and first exposure to human immunodeficiency virus type 1 (HIV-1) varies among individuals. Some individuals, termed long-term nonprogressors (LTNPRs), remain free from AIDS related complex (ARC) or AIDS for more than 10 years after infection without any antiretroviral therapy. Viral factors, host factors or both may contribute to this long-term nonprogression for AIDS [1]. HIV-1 *nef* gene has been suggested as one of the viral factors associated with the status of disease progression, as many LTNPRs specifically had gross deletions in *nef* [2–6]. Recently we reported by molecular virological analysis that *nef* is important for AIDS development [7]. In that study, we examined various activities of HIV-1 *nef*; (1) enhancement of viral infectivity, (2) down-regulation of CD4, (3) down-regulation of MHC-I, (4) binding ability with Hck [7]. The results obtained have demonstrated that augmentation of viral infectivity by *nef* is critically associated with AIDS [7].

Earlier studies on simian immunodeficiency virus (SIV) also show the importance of *nef* for simian AIDS [8,9]. In those reports, another accessory gene, *vif*, was found to be responsible for AIDS [8,10]. As for the potential role of HIV-1 *vif* for AIDS induction, few studies have been reported so far [11,12]. In the tissue culture system, Vif is known to confer infectivity on progeny virions in a producer cell-dependent manner, and producer cells are, therefore, divided into permissive and nonpermissive [13–16]. Viruses lacking *vif* fail to replicate in nonpermissive cells such as peripheral blood mononuclear cells (PBMCs), macrophages, and H9 cells. We have reported that various mutant *vif* clones show distinct growth phenotypes in H9 and semi-permissive A3.01

* Corresponding author. Tel.: +81-88-633-9232; fax: +81-88-633-7080.
E-mail address: mfujita@basic.med.tokushima-u.ac.jp (M. Fujita).

1286-4579/$ - see front matter © 2004 Elsevier SAS. All rights reserved.
doi:10.1016/j.micinf.2004.04.005

cells [17,18]. Hassaine et al. [11] examined the sequence of *vif* from LTNPRs and found that the amino acid at position 132 of Vif is associated with the viral load in LTNPRs. A recent report by Alexander et al. [12] described that a two-amino-acid insertion in Vif may contribute to the nonprogression of mother and child for AIDS. In this study, proviral reporter clones carrying *vif* from six LTNPRs or seven progressors (PRs) were generated, and their growth kinetics in nonpermissive and semi-permissive cells were examined. The results described here suggest that HIV-1 *vif* is important for the induction of AIDS.

2. Materials and methods

2.1. Study subjects

Samples from six Japanese LTNPRs (p1–p6) and seven PRs (p12–p18) were examined in the present study. The details of all study subjects have been described previously [19,20].

2.2. Cells

Lymphocytic cell lines H9 (ATCC HTB-176) and A3.01 [21] were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum as previously described [21,23]. A monolayer cell line 293T [22] was maintained in Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum as previously described [23].

2.3. Transfection and reverse transcription (RT) assay

For transfection of uncleaved plasmid DNA into lymphocytic H9 and A3.01 cells, the electroporation method was used as previously described [23]. For transfection of adherent 293T cells, the calcium–phosphate coprecipitation technique was used [23]. After electroporation, virus production in the culture supernatants was monitored at intervals by virion-associated reverse transcriptase (RT) activity as previously described [24].

2.4. Western immunoblotting

Cell lysates were prepared as described before [24], and proteins were resolved on sodium dodecyl sulfate–12.5% polyacrylamide gels, followed by electrophoretic transfer to polyvinylidene fluoride membranes (Immobilon-P, Millipore Co., Bedford, MA, USA). The membranes were treated with anti-Vif (NIH AIDS Research and References Reagent Program, catalog no. 2746) and anti-p24 [25] antibodies and visualized using an ECL plus Western blotting detection system (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).

2.5. Construction of basic and pilot clones

The wild-type (wt) infectious molecular clone of HIV-1, designated pNL432 (GenBank Accession no. AF324493), has been described previously [23]. For construction of basic clone pNL-SX, shown in Fig. 1, an appropriate DNA fragment of pNL432 was subcloned into pBluescipt SK(+) (Stratagene, La Jolla, CA, USA), and the mutations were introduced using QuikChange site-directed mutagenesis kit (Stratagene) as follows. *Sma*I and *Xba*I sites were generated at 5′ and 3′ ends of *vif*, respectively. Four methionine codons were changed to one valine and three threonine codons so as not to act as initiation codon. The mutated DNA fragment was cloned back into pNL432 to construct pNL-SX. To construct pilot clones carrying NLVif, NLΔ19Vif and NLΔVif, shown in Fig. 1 (pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif, respectively), wt and mutant *vif* sequences were amplified by polymerase chain reaction (PCR) with *Sma*I at the 5′ and *Xba*I at the 3′ ends. As templates, pNL432 was used for NLVif and NLΔ19Vif, and its frame-shift mutant pNL-Nd [26,27] was used for NLΔVif. Two oligonucleotide primer pairs used were as follows: 5′-TCCCCCGGGATGGAAAACAGATGGCAGGT-3′ (sense) and 5′-GCTCTAGACTAGTGTCCATTCATTGTATG-3′ (antisense) for NLVif and NLΔVif, 5′-TCCCCCGGGATGGAAAACAGATGGCAGGT-3′ (sense) and 5′-GCTCTAGACTATCTGTCCTCTGTCAGTTTC-3′ (antisense) for NLΔ19Vif. The PCR conditions were as follows: 94 °C for 45 s, 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and finally 72 °C for 10 min. The PCR-amplified DNA fragments were inserted into pNL-SX to construct various pilot clones pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif.

2.6. Proviral reporter constructs

Template DNAs from individuals for *vif* amplification in this study have been previously described [20]. Oligonucleotide primers and cycling conditions are essentially the same as described above for NLVif. The amplified DNA fragments were inserted into pNL-SX to obtain various reporter constructs.

3. Results and discussion

3.1. Establishment of an assay system for evaluating vif function

In this study, we analyzed the function of *vif* from individuals infected with HIV-1. As the ability to confer infectivity on virions is the most important function assigned to *vif* so far [13–16], we established a virological assay system based on a molecular clone modified from pNL432 [23]. As shown in Fig. 1A, we constructed a basic clone, designated pNL-SX, carrying *Sma*I and *Xba*I sites downstream of *pol* and upstream of *vpr*. We then amplified, by PCR, the wt *vif* sequence of pNL432 (NLVif), its shorter version of *vif* lacking 3′-57 bp (NLΔ19Vif), and its frame-shift *vif* mutant, which does not express most amino acid sequences of Vif

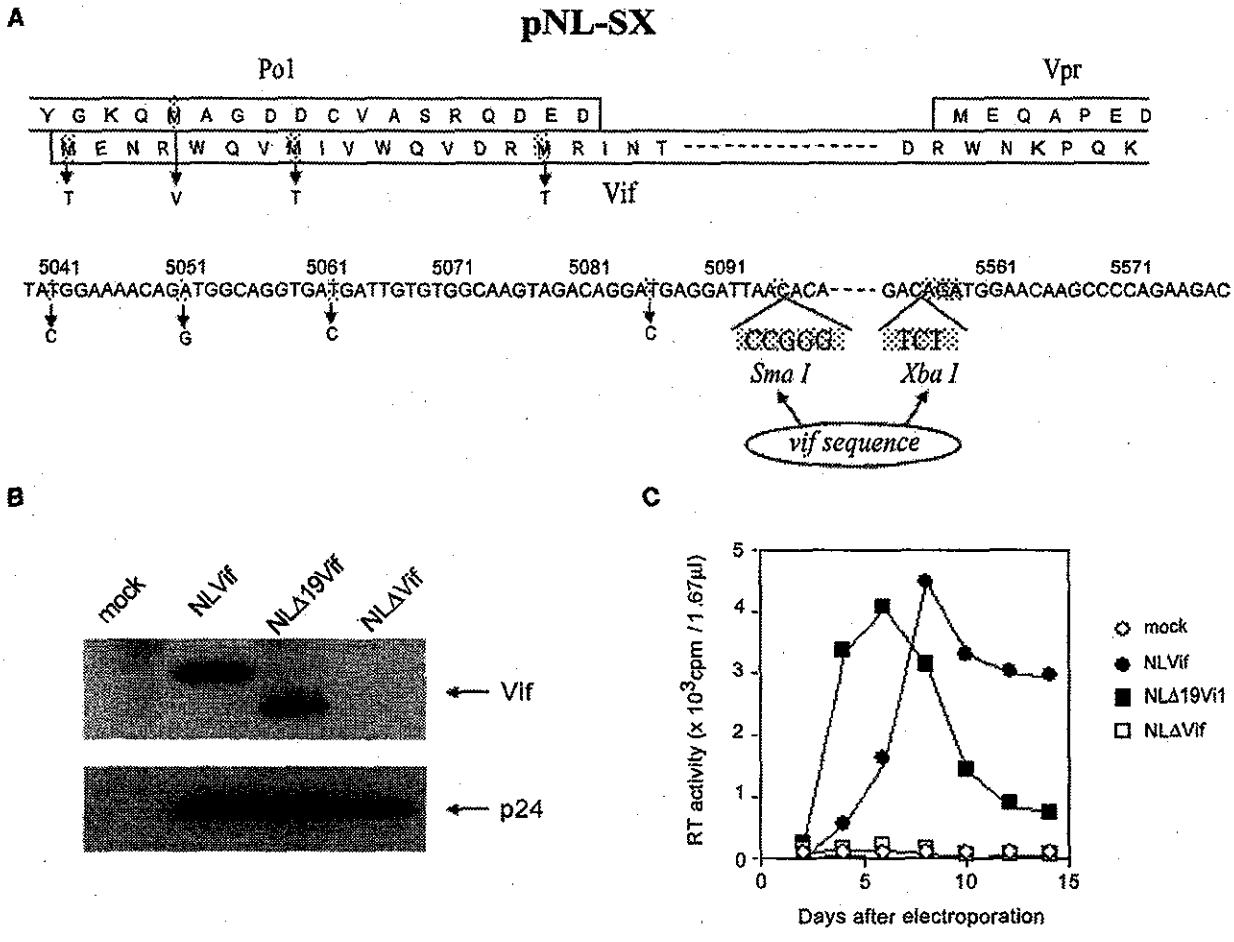


Fig. 1. Construction and characterization of the clones to determine activity of *vif* genes from individuals infected with HIV-1. (A) Schematic representation of the pNL-SX clone used in this study. To construct clone pNL-SX, four methionine (in *pol* and *vif* genes) codons of pNL432 (GenBank Accession no. AF324493) [23] were changed to one valine and three threonine codons so as not to act as initiation codon, and *Sma*I and *Xba*I sites were introduced as indicated. Various *vif* sequences with *Sma*I at 5′ and *Xba*I at 3′ ends were amplified by PCR using pNL432, its *vif* mutants, and DNAs from the individuals as templates. The amplified DNAs were inserted into pNL-SX to generate pilot and reporter clones. Amino acid (Pol, Vif and Vpr) and nucleotide sequences of pNL432 (nucleotide nos. 5040–5579) are shown. (B) Monitoring of Vif expression by Western blotting. 293T cells were transfected with the pilot proviral clones, as previously described [23], and cell lysates were prepared on day 2. Cell lysates were analyzed for the expression of Vif and Gag-p24 proteins by Western immunoblotting using appropriate antibodies. The three sequences inserted into pNL-SX were indicated at the top. NLVif, full-length *vif* sequences of pNL432 [23]; NLΔ19Vif, mutant *vif* sequences of NL432 lacking C-terminal 19 amino acids [28]; NLΔVif, mutant *vif* sequences of pNL432 carrying a frame-shift mutation at the *Nde*I site, which can encode only 28 amino acids of Vif sequence [26]. Mock, pUC19. (C) Growth kinetics in H9 cells of the pilot proviral clones. Cells were electroporated with 10 μg of the proviral clones [23], and RT production in the culture supernatants was determined, as previously described [24]. The same clones were used in (B) and (C).

(NLΔVif), and inserted these sequences into pNL-SX to construct pilot proviral clones pNL-SX/NLVif, pNL-SX/NLΔ19Vif and pNL-SX/NLΔVif. We and others have already reported that the C-terminal 19 amino acids of Vif (174–192) are dispensable without altering its functionality [28,29]. To confirm that pNL-SX is valid for virological assessment, the ability of these pilot clones to express Vif and to confer infectivity on virions was determined. These clones were transfected into 293T cells, and the expression of wt (NLVif) and the short version (NLΔ19Vif) of Vif was monitored by Western blotting. As can be clearly seen in Fig. 1B, wt and short Vif were readily detected. These clones were then electroporated into nonpermissive H9 cells, and RT production in the culture supernatants was monitored to determine their infectivity. As shown in Fig. 1C, viruses carrying wt (NL-SX/NLVif) and short type *vif* (NL-SX/NLΔ19Vif) could efficiently replicate in H9 cells, as expected, in contrast to the virus lacking functional *vif* (NL-SX/NLΔVif). These results indicated that pNL-SX can be used for evaluating the functional ability of *vif* from individuals infected with HIV-1.

3.2. Analysis of vif activity from LTNPRs and PRs

The pNL-SX system described above was used to evaluate the functionality of *vif* derived from six Japanese LTNPRs, who were free from ARC or AIDS for more than 10 years after infection, and from seven PRs [19,20]. *Vif* sequences in

Table 1
Activity of HIV-1 *nef* and *vif* genes derived from LTNPRs and PRs of AIDS

Individual [a]	*nef* [b]	*vif*		
	MAGI infectivity	Clone [c]	Growth [d] in	
			H9	A3.01
p1 LTNPR (<1.0)	m,m	10	–	–
		12-a	+	+
		12-b	+	+
		12-c	+	+
		12-d	+	+
		13	+	+
		14	+	+
			0.86	0.86
p2 LTNPR (<1.0)	nd	1	+	+
		4	+	+
		5-a	+	+
		5-b	+	+
			1.00	1.00
p3 LTNPR (<1.0)	m,–	1	–	–
		3	+*	+*
		4	–	–
		5	+	+
		6	+	+
			0.60	0.60
p4 LTNPR (<1.0)	m,m	1	–	–
		2	–	–
		3	+	+
		4	+	+
		5	+	+*
			0.60	0.60
p5 LTNPR (<1.0)	nd	1	–	–
		3	–	–
		4	–	–
		9	–	–
			0.00	0.00
p6 LTNPR (<1.0)	m,m	c-a	–	–
		c-b	–	–
		g	+	+
		h	+	+
		i	–	–
			0.40	0.40
p12 PR	nd	7	+	+
(2.2)		8-a	–	–
		8-b	–	–
		9	–	–
		10	–	–
			0.20	0.20
p13 PR	+	1	+	+
(2.4)		2-a	+	+
		2-b	+	+
		7	+	+
			1.00	1.00
p14 PR	+,+	3	–	+*
(20)		5	+	+*
		6	+	+
		7	–	–
			0.50	0.75
p15 PR	+,+	1	+	+
(88)		2	+	+
		4	+	+
		5	–	+
		6	+	+
			0.80	1.00
p16 PR	+,+	1	+	+
(29)		2	+	+
		3	+	+
		4	+	+
		5	+	+
			1.00	1.00
p17 PR	+	1	+	+
(21)		3	+	+
		4	+	+
		5	+	+
			1.00	1.00
p18 PR	+	1	+	+
(3.8)		2	+	+
		4	+	+
			1.00	1.00

[a] Identification numbers of individuals, stage of disease progression, and viral RNA level in the plasma ($\times 10^4$ copies per ml), in parentheses, are indicated [19].

[b] Infectivity of the clones based on NL432 carrying *nef* sequences derived from the individuals (one or two clones per person) was determined by MAGI assay [7]. Infectivity of the clones relative to that of the clone carrying NL432 *nef* is shown. +, >60%; –, <30%; m, 30–60%; nd, not done.

[c] (The same number or the same alphabet)-(alphabet) indicates that the clones carry the same *vif* sequences. For example, three and four different *vif* sequences have been identified in p2 and p6, respectively.

[d] Growth ability in H9 and A3.01 cells of viral clones derived from pNL-SX (Fig. 1A) carrying *vif* genes from the individuals. Growth phenotype of the clones similar to that of NL-SX/NLVif and that of NL-SX/NLΔVif (Fig. 2A) is indicated by + and –, respectively. Growth phenotype of the viruses which grew more poorly than NL-SX/NLVif but better than NL-SX/NLΔVif is indicated by +* (Fig. 2A). The ratio of the number of clones with active (+ and +*) *vif* to that of clones examined is indicated on the last line.

these individuals were amplified by PCR and inserted into pNL-SX to construct 53 reporter clones (Table 1). Nonpermissive H9 and semi-permissive A3.01 cells were used to examine the growth ability of all clones generated, as mutant *vif* clones show distinct growth phenotypes in these two cell lines [17,18]. Representative growth kinetics of the clones are shown in Fig. 2A. In these experiments, clones carrying wt (NLVif) and Δ*vif* (NLΔVif) served as positive and negative controls, respectively. Based on the growth phenotypes, various *vif* sequences were considered to be active or inactive. Clones p1-12, p3-3 and p13-1 were thought to be active, while clones p1-10, p3-4, p14-3 and p14-7 were inactive as judged by virus replication in H9 cells (Fig. 2A). In A3.01 cells, clones p1-12, p3-3, p13-1 and p14-3 were active, while p1-10, p3-4, and p14-7 were inactive (Fig. 2A). The functionality of all *vif* sequences from LTNPRs and PRs is summarized in Table 1. Two clones, p14-3 and p15-5, showed

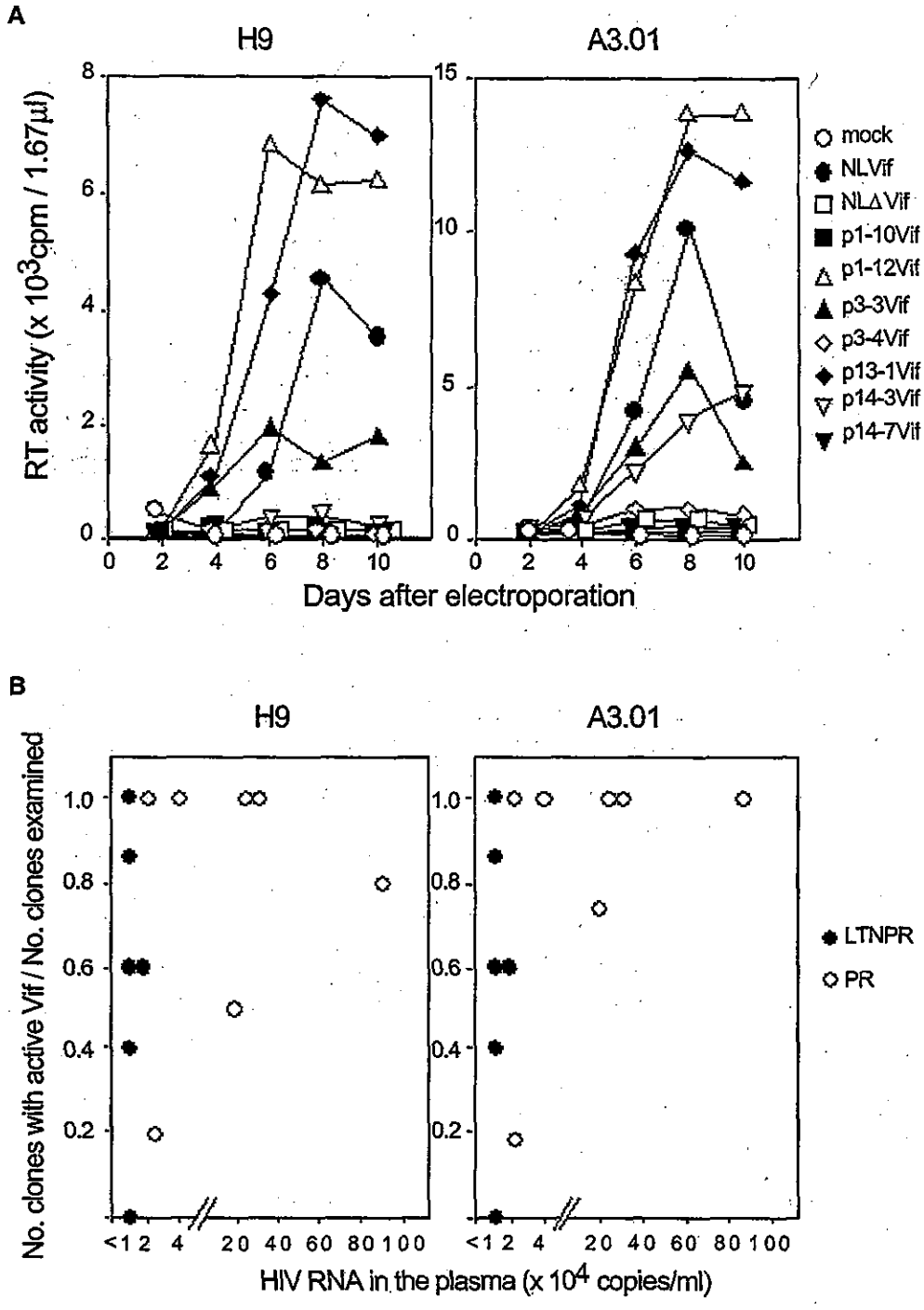


Fig. 2. Activity of HIV-1 *vif* genes derived from LTNPRs and PRs for AIDS. (A) Growth kinetics in H9 and A3.01 cells of various clones carrying *vif* sequence from the individuals. Cells were electrophorated, as previously described [23], with 10 μg of proviral clones carrying the *vif* sequences indicated on the right, and RT production in the culture supernatants at intervals was determined, as previously described [24]. Selected samples are shown in the figure for clarity (all the data are presented in Table 1). The clones were constructed by insertion of the *vif* sequences derived from the individuals into *Sma*I and *Xba*I sites of pNL-SX (Fig. 1A). The *vif* sequences from the individuals are indicated by the individual no.-clone no. Sequences NLVif and NLΔVif (for details, see the legend to Fig. 1) were used as positive and negative controls, respectively. Mock, pUC19. (B) Ratio of active and inactive *vif* clones in LTNPRs and PRs for AIDS. The ratio of clones with active *vif* in each person versus viral RNA level in his/her plasma (10^4 copies per ml) [19] is plotted for all the clones examined (Table 1).

distinct growth phenotypes in H9 and A3.01 cells (Fig. 2A, Table 1).

The ratio of active *vif* clones (in both cell lines) to the total number of clones screened per individual is also shown in Table 1. When the ratio versus the viral load in plasma (Table 1) was plotted, results as shown in Fig. 2B were obtained. LTNPRs enrolled in the present study had varied ratios of active *vif* clones, ranging from 0 to 1.0. The results from the majority of LTNPRs indicate no apparent relationship between presence or absence of functional *vif* and long-

term nonprogression of infection. Subject p5 was an exception and had inactive *vif* in all the clones screened. This finding suggested a possibility that a defect of *vif* function in p5 could be responsible for the long-term nonprogression. On the other hand, all PRs with high viral load (HIV RNA $>20\times10^4$ copies per ml) had high ratios (0.5 or >0.5) of active *vif* clones. We and others have reported that primary cultured PBMCs and macrophages are non or less permissive for HIV virions lacking *vif* [30–35]. Recently, molecular biological demonstration has shown that *vif* confers infectivity on virions in nonpermissive cells. Vif counteracts an endogenous cellular factor, APOBEC3G, that inhibits HIV-1 replication [36,37]. It is well anticipated that the activity of *vif* modulates HIV-1 replication in individuals infected with virus.

3.3. Conclusion

Table 1 summarizes the major data in our two papers (reference [7] and this article). In these two reports, *nef* and *vif* from the same individuals were systematically analyzed for their potential roles in the development of AIDS. The *nef* genes from LTNPRs were found to have lower activity to enhance viral infectivity than those from PRs. The results of our *nef* and *vif* studies have shown that all PRs with a high viral load have a high ratio of active genes, strongly suggesting active *vif* and *nef* are required for HIV-1 replication in individuals. The *vif* and *nef* genes, therefore, would influence the progression and outcome of the disease in infected individuals. This is consistent with the previous reports analyzing *vif* sequences in disease progression [11,12]. On the other hand, the active/inactive ratio of *vif* varied in the samples from LTNPRs (from 0 to 1.0), different from that of *nef*. Without active *nef*, infected individuals appeared not to develop ARC or AIDS. In tissue cultures, *nef* is critical for HIV replication in resting PBMC [38,39] but not in stimulated PBMC or macrophages [32,35]. The *vif* is known to be indispensable for virus replication in these cells [30–35]. Because *vif* is more essential in virus replication than *nef*, it is reasonable to assume that *vif*-inactive viruses cannot or hardly persist in individuals. Further study is necessary to draw a clear conclusion regarding the functional importance of *vif* for disease progression.

Acknowledgments

We thank Kazuko Yoshida for editorial assistance. Antibody to HIV-1 Vif was obtained though the NIH AIDS Research and Reference Reagent Program (Catalog no. 2746). This work was supported in part by a Grant-in-Aid for Scientific Research (B) (14370103) and a Grant-in Aid for Scientific Research (C) (15590420) from the Japan Society for the Promotion of Science, and a Health Sciences Research Grant (Research on HIV/AIDS 13110201) from the Ministry of Health, Labor and Welfare of Japan.

References

[1] O.J. Cohen, A.S. Fauci, Pathogenesis and medical aspects of HIV-1 infection, in: fourth ed, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, 2, Lippincott Williams & Wilkins, Philadelphia, 2001, pp.2043–2094.

[2] N.J. Deacon, A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D.J. Hooker, D.A. McPhee, A.L. Greenway, A. Ellett, C. Chatfield, V.A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J.S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, J. Mills, Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients, Science 270 (1995) 988–991.

[3] F. Kirchhoff, T.C. Greenough, D.B. Brettler, J.L. Sullivan, R.C. Desrosiers, Absence of intact *nef* sequences in a long-term survivor with nonprogressive HIV-1 infection, New Engl. J. Med. 332 (1995) 228–232.

[4] J.C. Learmont, A.F. Geczy, J. Mills, L.J. Ashton, C.H. Raynes-Greenow, R.J. Garcia, W.B. Dyer, L. McIntyre, R.B. Oelrichs, D.I. Rhodes, N.J. Deacon, J.S. Sullivan, D.A. McPhee, S. Crowe, A.E. Solomon, C. Chatfield, S. Blasdall, H. Kuipers for the Sydney Blood Bank Cohort Research Group, Immunologic and virologic status after 14–18 years of infection with an attenuated strain of HIV-1—a report from the Sydney blood bank cohort, New Engl. J. Med. 340 (1999) 1715–1722.

[5] D.I. Rhodes, L. Ashton, A. Solomon, A. Carr, D. Cooper, J. Kaldor, N. Deacon for the Australian Long-Term Nonprogressor Study Group, Characterization of three *nef*-defective human immunodeficiency virus type 1 strains associated with long-term nonprogression, J. Virol. 74 (2000) 10581–10588.

[6] R. Salvi, A.R. Garbuglia, A.D. Caro, S. Pulciani, F. Montella, A. Benedetto, Grossly defective *nef* gene sequences in a human immunodeficiency virus type 1-seropositive long-term nonprogressor, J. Virol. 72 (1998) 3646–3657.

[7] M. Tobiume, M. Takahoko, T. Yamada, M. Tatsumi, A. Iwamoto, M. Matsuda, Inefficient enhancement of viral infectivity and CD4 downregulation by human immunodeficiency virus type 1 Nef from Japanese long-term nonprogressors, J. Virol. 76 (2002) 5959–5965.

[8] R.C. Desrosiers, J.D. Lifson, J.S. Gibbs, S.C. Czajak, A.Y.M. Howe, L.O. Arthur, R.P. Johnson, Identification of highly attenuated mutants of simian immunodeficiency virus, J. Virol. 72 (1998) 1431–1437.

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

[10] J.S. Gibbs, D.A. Regier, R.C. Desrosiers, Construction and in vitro properties of SIVmac mutants with deletions in "nonessential" genes, AIDS Res. Hum. Retroviruses 10 (1994) 607–616.

[11] G. Hassaine, I. Agostini, D. Candotti, G. Bessou, M. Caballero, H. Agut, B. Autran, Y. Barthalay, R. Vigne, the French ATL study group, Characterization of human immunodeficiency virus type 1 *vif* gene in long-term asymptomatic individuals, Virology 276 (2000) 169–180.

[12] L. Alexander, M.J. Aquino-DeJesus, M. Chan, W.A. Andiman, Inhibition of human immunodeficiency virus type 1 (HIV-1) replication by a two-amino-acid insertion in HIV-1 Vif from a nonprogressing mother and child, J. Virol. 76 (2002) 10533–10539.

[13] S. Bour, K. Strebel, HIV accessory proteins: multifunctional components of a complex system, Adv. Pharmacol. 48 (2000) 75–120.

[14] B.R. Cullen, HIV-1 auxiliary proteins: making connections in a dying cell, Cell 93 (1998) 685–692.

[15] M. Emerman, M.H. Malim, HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology, Science 280 (1998) 1880–1884.

[16] R. Inubushi, M. Tamaki, R. Shimano, A.H. Koyama, H. Akari, A. Adachi, Functional roles of HIV accessory proteins for viral replication, Int. J. Mol. Med. 2 (1998) 429–433.

[17] M. Fujita, A. Sakurai, N. Doi, M. Miyaura, A. Yoshida, K. Sakai, A. Adachi, Analysis of the cell-dependent replication potentials of human immunodeficiency virus type 1 *vif* mutants, Microbes Infect. 3 (2001) 1093–1099.

[18] M. Fujita, A. Sakurai, A. Yoshida, S. Matsumoto, M. Miyaura, A. Adachi, Subtle mutations in the cysteine region of HIV-1 Vif drastically alter the viral replication phenotype, Microbes Infect. 4 (2002) 621–624.

[19] T. Yamada, A. Iwamoto, Expression of a novel Nef epitope on the surface of HIV type 1-infected cells, AIDS Res. Hum. Retroviruses 15 (1999) 1001–1009.

[20] T. Yamada, A. Iwamoto, Comparison of proviral accessory genes between long-term nonprogressors and porogressors of human immunodeficiency virus type 1 infection, Arch. Virol. 145 (2000) 1021–1027.

[21] T. Folks, S. Benn, A. Rabson, T. Theodore, M.D. Hoggan, M. Martin, M. Lightfoote, K. Sell, Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immune deficiency syndrome (AIDS)-associated retrovirus, Proc. Natl. Acad. Sci. USA 82 (1985) 4539–4543.

[22] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 317 (1985) 169–171.

[23] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.

[24] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.

[25] H. Akari, T. Fukumori, A. Adachi, Cell-dependent requirement of human immunodeficiency virus type 1 gp41 cytoplasmic tail for Env incorporation into virions, J. Virol. 74 (2000) 4891–4893.

[26] A. Adachi, N. Ono, H. Sakai, K. Ogawa, R. Shibata, T. Kiyomasu, H. Masuike, S. Ueda, Generation and characterization of the human immunodeficiency virus type 1 mutants, Arch. Virol. 117 (1991) 45–58.

[27] H. Sakai, R. Shibata, J.I. Sakuragi, S. Sakuragi, M. Kawamura, A. Adachi, Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles, J. Virol. 67 (1993) 1663–1666.

[28] M. Fujita, S. Matsumoto, A. Sakurai, N. Doi, M. Miyaura, A. Yoshida, A. Adachi, Apparent lack of *trans*-dominant negative effects of various *vif* mutants on the replication of HIV-1, Microbes Infect. 4 (2002) 1203–1207.

[29] C. Ochsenbauer, V. Bosch, I. Oelze, U. Wieland, Unimpaired function of a naturally occurring C terminally truncated *vif* gene product of human immunodeficiency virus type 1, J. Gen. Virol. 77 (1996) 1389–1395.

[30] M. Fujita, A. Sakurai, A. Yoshida, M. Miyaura, A.H. Koyama, K. Sakai, A. Adachi, Amino acid residues 88 and 89 in the central hydrophilic region of human immunodeficiency virus type 1 Vif are critical for viral infectivity by enhancing the steady-state expression of Vif, J. Virol. 77 (2003) 1626–1632.

[31] D.H. Gabuzda, K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W.A. Haseltine, J. Sodroski, Role of *vif* in replication of human immunodeficiency virus type 1 in CD4$^+$ T lymphocytes, J. Virol. 66 (1992) 6489–6495.

[32] M. Kawamura, T. Ishizaki, A. Ishimoto, T. Shioda, T. Kitamura, A. Adachi, Growth ability of human immunodeficiency virus type 1 auxiliary gene mutants in primary blood macrophage cultures, J. Gen. Virol. 75 (1994) 2427–2431.

[33] U. Schwedler, J. Song, C. Aiken, D. Trono, *vif* is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells, J. Virol. 67 (1993) 4945–4955.

[34] J.H.M. Simon, M.H. Malim, The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes, J. Virol. 70 (1996) 5297–5305.

[35] K. Tokunaga, A. Ishimoto, K. Ikuta, A. Adachi, Growth ability of auxiliary gene mutants of human immunodeficiency virus types 1 and 2 in unstimulated peripheral blood mononuclear cells, Arch. Virol. 142 (1997) 177–181.

[36] B.R. Cullen, HIV-1 Vif: counteracting innate antiretroviral defenses, Mol. Ther. 8 (2003) 525–527.

[37] A.M. Sheehy, N.C. Gaddis, J.D. Choi, M.H. Malim, Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein, Nature 418 (2002) 646–650.

[38] M.D. Miller, M.T. Warmerdam, I. Gaston, W.C. Greene, M.B. Feinberg, The human immunodeficiency virus-1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages, J. Exp. Med. 179 (1994) 101–113.

[39] C.A. Spina, T.J. Kwoh, M.Y. Chowers, J.C. Guatelli, D.D. Richman, The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes, J. Exp. Med. 179 (1994) 115–123.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 20, Number 12, 2004, pp. 1352–1357

Sequence Note

Genetic Diversity of HIV Type 1 in Likasi, Southeast of the Democratic Republic of Congo

KAYOKO KITA,[1] NICAISE NDEMBI,[1] MICHEL EKWALANGA,[2] EIJI IDO,[3] RIGOBERT KAZADI,[2] BLAISE BIKANDOU,[4] JUN TAKEHISA,[1] TAICHIRO TAKEMURA,[3] SEIJI KAGEYAMA,[1] JUNJI TANAKA,[5] HENRI JOSEPH PARRA,[4] MASANORI HAYAMI,[3] and HIROSHI ICHIMURA[1]

ABSTRACT

To investigate the prevalence of subtypes A and C, and the existence of recombinants of both subtypes in the southeast of the Democratic Republic of Congo (DRC), blood samples were collected from 27 HIV-infected individuals in Likasi, located in an area bordering close to Zambia, and analyzed phylogenetically. Out of the 24 strains with a positive PCR profile for *pol*-IN and *env*-C2V3, 15 (62.5%) had a discordant subtype or CRF designation: one subtype A/G (*pol/env*), four A/U (unclassified), three G/A, one G/CRF01, three H/A, one J/C, one CRF02 (G)/A, and one U/A. Nine (37.5%) strains had a concordant subtype or CRF designation: five subtype A, two C, one D, and one CRF02/G. The remaining three samples negative for PCR with *env*-C2V3 primers used in this study were further analyzed with *env*-gp41 primers and revealed the presence of two profiles: two J/J (*pol*-IN/*env*-gp41) and one C/G. These data highlight the presence of a high proportion (16/27, 59.3%) of recombinant strains and a low prevalence (4.1 and 7.4%) of subtype C based on *env*-C2V3 and *pol*-IN analyses, respectively, in Likasi. In addition, this is the first report that CRF02_AG exists in DRC, though the epidemiological significance of the existence of CRF02_AG in DRC remains unknown.

HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) has been classified into three major phylogenetic groups, termed M (major), N (non-M, non-O), and O (outlier).[1] The vast majority of variants found worldwide and responsible for the AIDS pandemic belong to group M.[2,3] Phylogenetic analysis of group M has further led to its subclassification into nine pure subtypes, A–D, F–H, J, and K and subsubtypes A1, A2, F1, and F2.[2] Recently, it was realized that a significant fraction of HIV-1 isolates, 10–40% or more, exhibit a shift in subtypes when different regions of their genome are analyzed.[3] Currently, some of the mosaic HIV-1 genomes play a major role in the global AIDS epidemics and are designated as circulating recombinant forms (CRFs), CRF01-CRF16. Although subtypes A, C, and CRF02_AG are most prevalent in Africa, the distribution of CRF/subtype is very heterogeneous.[2–7] The proportion of CRF02_AG among subtype A strains based on *env* sequences decreases from west to central Africa, with an absence of CRF02_AG in the Democratic Republic of Congo (DRC).[2,8,9] The profile of HIV-1 endemic in DRC, such as high number of cocirculating HIV-1 subtypes, possible recombinant viruses, and unclassified strains, is consistent with that of an old and mature epidemic of HIV-1.[5]

The DRC is bordered on the southeast by Zambia. The majority (95%) of the HIV-1 strains circulating in Zambia are subtype C, although HIV-1 group M subtypes A, D, G, and J as well as group O have been identified.[2,3,10,11]

[1]Department of Viral Infection and International Health, Graduate School of Medical Science, Kanazawa University, Kanazawa 920-8640, Japan.
[2]Laboratoire de virologie, INRB, Kinshasa, Republique Democratique du Congo.
[3]Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan.
[4]Cité Louis Pasteur-Laboratoire National de Santé Publique, Brazaville 120, Congo.
[5]Department of Laboratory Science, School of Health Sciences, Kanazawa University, Kanazawa 920-8640, Japan.