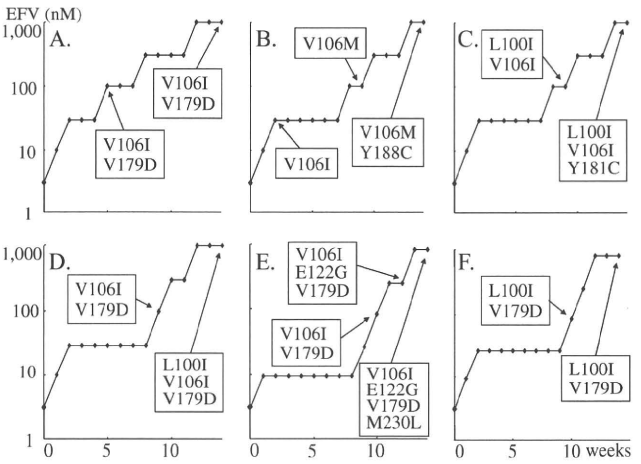

FIG. 1. Selected amino acid substitutions under selective pressure from EFV. HIV-1_{V106I} (A to C) and HIV-1_{V179D} (D to F) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The selected amino acid substitutions were analyzed at several passages by sequencing the proviral HIV-1 RT coding region in MT-2 cells. Amino acid substitutions compared with wild-type strain BH 10 are shown.

listed as EFV and NVP resistance-associated mutations and V90I, K101E, V106I, E138A, V179D, and V179T are listed as ETV resistance-associated mutations in the Drug Resistance Mutation List of the International AIDS Society (IAS)-USA (14). Of these NNRTI resistance-associated mutations, V106I (2.5%) and V179D (5.8%) were frequently observed in treatment-naïve patients, while the other six mutations were less common (0.3 to 0.8%). These data indicate that V106I and V179D occur naturally in treatment-naïve individuals at significant frequencies and are clinically important as polymorphic mutations. Accordingly, we focused on these two polymorphic mutations and analyzed their effects on the development of NNRTI resistance. There was no overt linkage among the amino acids at the positions of NNRTI resistance-associated mutations, though one patient harbored both V106I and V179D without any other resistance-associated mutations. There was no correlation between clades and the 106th and 179th amino acids.

Selection of EFV-resistant HIV-1 from HIV-1_{V106I} and HIV-1_{V179D}. To analyze the effects of V106I and V179D on the resistance pattern of mutations, EFV-resistant HIV-1 strains were selected from monoclonal HIV-1 strains harboring V106I (HIV-1_{V106I}) or V179D (HIV-1_{V179D}). These selection experiments were performed independently in triplicate. Interestingly, in one of three selection experiments with HIV-1_{V106I}, V179D emerged when the EFV concentration reached 100 nM, and it was persistently identified until the end of the passages without additional mutations (Fig. 1A). In two other experiments with HIV-1_{V106I}, I106M emerged, followed by Y188C, and L100I emerged, followed by Y181C. These four mutations are already known NNRTI resistance-associated mutations (Fig. 1B and C) (1, 14, 25). In one of three experiments with HIV-1_{V179D}, V106I emerged when the EFV concentration reached 100 nM, and L100I further emerged at the end of the experiments (Fig. 1D). In another experiment, V106I emerged when the EFV concentration was 100 nM, and E122G and M230L followed subsequently (Fig. 1E). In the last experiment, L100I emerged when the EFV concentration reached 100 nM, and it remained until the end of the passages without additional mutations (Fig. 1F). In summary, selected by EFV, V179D emerged in one of three experiments from HIV-1_{V106I} and V106I emerged in two of three experiments from HIV-1_{V179D}, suggesting that the combination of two polymorphic mutations, V106I and V179D, alters viral susceptibility to EFV.

NNRTI susceptibility of recombinant HIV-1 strains. To analyze the effects of V106I, V179D, and their combination on NNRTI susceptibility, a panel of recombinant HIV-1 clones was constructed and their EFV, NVP, and ETV EC_{50}s were determined. As expected, the single mutation V106I or V179D did not confer significant resistance to EFV and NVP (Table 2). HIV-1_{V106A} was generated as a reference, and it showed high-fold resistance to NVP but not to EFV, in agreement with previous studies (10, 14). The addition of V179D to HIV-1_{V106A} (HIV-$1_{V106A/V179D}$) increased its resistance to NVP and conferred significant resistance to EFV. The combination of the two polymorphic mutations, V106I and V179D, which emerged in resistance selection experiments with EFV, conferred significant resistance not only to EFV but also to NVP. In the susceptibility assay with ETV, it exhibited potent anti-HIV-1 activity in all of the HIV-1 strains examined, including the NNRTI-resistant clones described above, indicating that ETV has a different binding formulation with RT molecules than EFV and NVP do.

Replication kinetics of recombinant HIV-1 strains. To analyze the effects of single mutations and their combinations on HIV-1 replication efficiency, we assayed the replication kinetics of recombinant HIV-1 strains in MT-2 cells in the absence or presence of an NNRTI. Each replication assay was performed in triplicate and repeated three times. In the absence of

TABLE 2. NNRTI susceptibility of recombinant HIV-1 strains

HIV-1 strain	Mean EC_{50} (μM) ±SD (fold resistance)[a]		
	EFV	NVP	ETV
Wild type	0.002 ± 0.0007	0.05 ± 0.01	0.0012 ± 0
V106A clone	0.003 ± 0.0009 (1.5)	3.43 ± 0.98 (69)	0.0005 ± 0.0001 (0.40)
V106I clone	0.003 ± 0.0003 (1.5)	0.02 ± 0.0012 (0.40)	0.0015 ± 0.0004 (1.3)
V179D clone	0.004 ± 0.0002 (2.0)	0.13 ± 0.02 (2.6)	0.0019 ± 0.0004 (1.6)
V106A V179D clone	0.013 ± 0.004 (6.5)	4.53 ± 0.72 (91)	0.0014 ± 0.0004 (1.2)
V106I V179D clone	0.029 ± 0.007 (15)	0.37 ± 0.12 (7.0)	0.0024 ± 0.0004 (2.0)

[a] The drug susceptibility assay was performed in triplicate and repeated three times (nine experiments). Data are means of nine experiments.

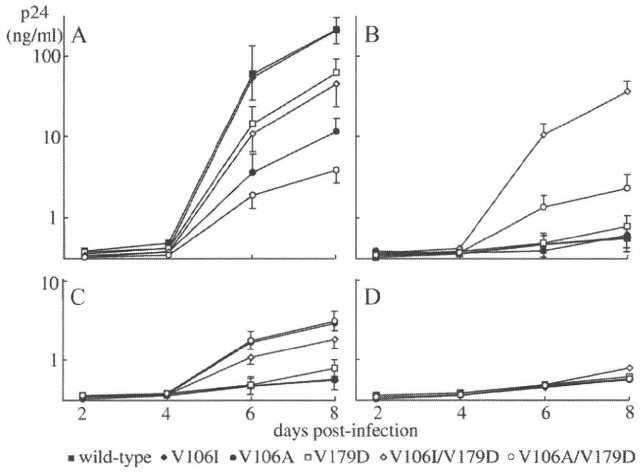

FIG. 2. Replication kinetics of recombinant HIV-1 clones in the absence and presence of NNRTIs. Recombinant HIV-1 clones were propagated in MT-2 cells in the absence (A) and presence of 10 nM EFV (B), 100 nM NVP (C), or 10 nM ETV (D). The concentration of p24 in the culture medium was measured every other day. The assay was performed in triplicate and repeated three times (nine experiments). The data are logarithmic mean p24 concentrations ± standard deviations (days 6 and 8 in panels A to C).

an NNRTI, V106I did not alter HIV-1_{WT} replication while V179D significantly reduced HIV-1_{WT} replication (p24 of HIV-1_{V179D} versus HIV-1_{WT} on day 8; $P < 0.05$) (Fig. 2A). The addition of V106I to HIV-1_{V179D} (HIV-$1_{V106I/179D}$) did not significantly alter its replication kinetics. V106A significantly reduced viral replication (p24 of HIV-1_{V106A} versus HIV-1_{WT} on day 8; $P < 0.01$), and the addition of V179D to it (HIV-$1_{V106A/179D}$) further reduced the virus's replication ability (p24 of HIV-$1_{V106A/V179D}$ versus HIV-1_{V106A} on day 8; $P < 0.05$).

In the presence of 10 nM EFV, HIV-1_{WT}, HIV-1_{V106I}, and HIV-1_{V106A} failed to propagate and HIV-1_{V179D} exhibited reduced replication compared with that observed in the absence of an NNRTI ($P < 0.01$) (Fig. 2B). HIV-$1_{V106I/V179D}$ and HIV-$1_{V106A/V179D}$ showed efficient replication, though the replication of HIV-$1_{V106A/V179D}$ was slightly reduced compared with that observed in the absence of an NNRTI. In the presence of 100 nM NVP, HIV-1_{WT} and HIV-1_{V106I} failed to propagate and HIV-1_{V179D} exhibited reduced replication compared with that observed in the absence of an NNRTI ($P < 0.01$) (Fig. 2C). HIV-1_{V106A} and HIV-$1_{V106I/V179D}$ showed efficient replication, though the replication of HIV-$1_{V106I/V179D}$ was reduced significantly compared with that observed in the absence of an NNRTI ($P < 0.05$). HIV-$1_{V106A/V179D}$ exhibited replication comparable to that observed in the absence of an NNRTI. In the presence of 10 nM ETV, all of the HIV-1 strains examined exhibited severely compromised replication (Fig. 2D). The results of the replication kinetics experiments were in agreement with the drug susceptibility data.

To analyze the precise roles of V106I and V179D in HIV-$1_{V106I/V179D}$, a competitive HIV-1 replication assay was performed using H9 cells (7, 17). The assay of HIV-1_{V106I} and HIV-$1_{V106I/V179D}$ indicated that the addition of V179D compromised the replication fitness of HIV-1_{V106I} but conferred resistance to EFV and NVP (see Fig. S1A to C in the supplemental material). The assay of HIV-1_{V179D} and HIV-$1_{V106I/V179D}$ indicated that the addition of V106I slightly reduced the replication ability of HIV-1_{V179D} but conferred resistance to EFV and NVP (see Fig. S1D to F in the supplemental material). In the presence of 10 nM ETV, the HIV-1 clones examined could not be passaged efficiently because ETV efficiently suppressed viral replication.

NNRTI susceptibility of HIV-1 clinical isolates. Analysis of the recombinant HIV-1 clones indicated that the combination of V106I and V179D conferred significant resistance to EFV and NVP but not to ETV, although each single mutation did not alter viral susceptibility to NNRTIs (Table 2). To determine the clinical relevance of the results of recombinant HIV-1 analysis, HIV-1 clinical isolates were obtained using MAGIC-5 cells from seven treatment-naïve individuals (cases 1 to 7). In cases 1 and 2, only wild-type amino acids (valine) were detected at the 106th and 179th codons of the HIV-1 RT coding region. In cases 3 and 4, V106I was detected and wild-type valine was found at the 179th codon. In cases 5 and 6, wild-type valine was found at the 106th codon and V179D was identified. In case 7, both V106I and V179D were identified, and none of the other patients harbored both mutations in HIV-1 RT. Subclonal analysis determined that V106I and V179D were on the same virus and that they were highly dominant in case 7 (Table 1). All seven of the patients were infected with HIV-1 subtype B, and no other known resistance-associated mutations were detected at any RT codon other than the 106th and 179th. The six isolates derived from cases 1 to 6 did not show significant resistance to NNRTIs (Table 3). The isolate from case 7, however, exhibited significant resistance to EFV and

TABLE 3. NNRTI susceptibility of clinical HIV-1 isolates

HIV-1 strain (case no.)	Mean EC_{50} (μM) ±SD (fold resistance)[a]		
	EFV	NVP	ETV
Wild type (V106 V179)	0.002 ± 0.0001	0.05 ± 0.004	0.0013 ± 0.0001
V106 V179 isolate (1)	0.002 ± 0.0004 (1.0)	0.04 ± 0.004 (0.8)	0.002 ± 0.0004 (1.5)
V106 V179 isolate (2)	0.002 ± 0.0003 (1.0)	0.04 ± 0.004 (0.8)	0.003 ± 0.0002 (2.3)
V106I V179 isolate (3)	0.002 ± 0.0002 (1.0)	0.03 ± 0.01 (0.6)	0.0012 ± 0.0002 (0.9)
V106I V179 isolate (4)	0.004 ± 0.001 (2.0)	0.09 ± 0.01 (1.8)	0.0024 ± 0.0002 (1.8)
V106 V179D isolate (5)	0.006 ± 0.001 (3.0)	0.07 ± 0.02 (1.4)	0.0015 ± 0.0002 (1.2)
V106 V179D isolate (6)	0.004 ± 0.002 (2.0)	0.07 ± 0.004 (1.4)	0.0011 ± 0.0001 (0.8)
V106I V179D isolate (7)	0.01 ± 0.001 (7.0)	0.19 ± 0.02 (3.8)	0.002 ± 0.0003 (1.5)

[a] The drug susceptibility assay was performed in triplicate and repeated three times (nine experiments). Data are means of nine experiments.

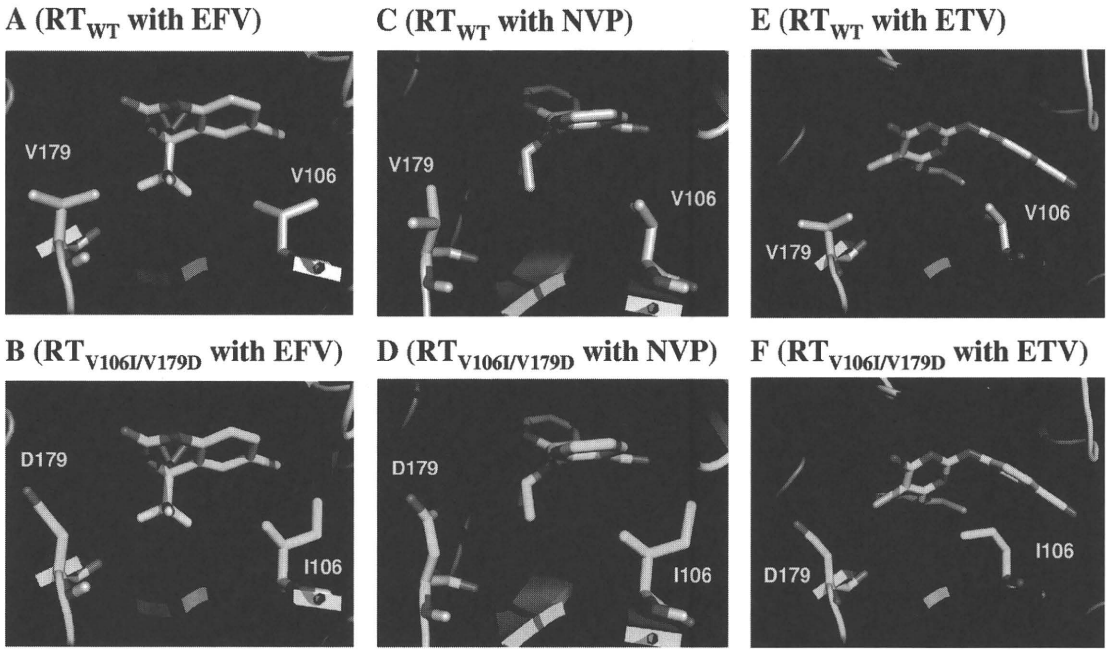

FIG. 3. Interactions of NNRTIs with the 106th and 179th residues of RTs. Interaction sites of NNRTIs and RTs in the models are shown. (A) Wild-type RT (RT_{WT}) with EFV. (B) $RT_{V106I/V179D}$ with EFV. (C) RT_{WT} with NVP. (D) $RT_{V106I/V179D}$ with NVP. (E) RT_{WT} with ETV. (F) $RT_{V106I/V179D}$ with ETV. In the RT_{WT} and $RT_{V106I/V179D}$ models, carbon atoms appear gray and cyan, respectively. NNRTIs and the 106th and 179th residues are highlighted by the stick configuration. Blue sticks, nitrogen; red sticks, oxygen; light blue, fluorine; light green, chlorine; pink sticks, bromine atoms.

NVP but not to ETV. These data confirmed the results obtained by recombinant HIV-1 analysis.

Structural modeling analysis. To obtain structural insight into the molecular mechanisms through which RT mutations alter susceptibility to NNRTIs, we conducted structural analyses by computational methods. A total of 18 structural models of RT-NNRTI complexes were constructed with six RTs (wild-type RT and V179D, V106A, V106I, V106A/V179D, and V106I/V179D mutant RTs) and three NNRTIs (EFV, NVP, and ETV), and the differences in binding energy between the mutant and wild-type complexes ($\Delta\Delta G_b$) were calculated. Notably, $\Delta\Delta G_b$ was proportionally related to the logarithm of fold resistance in RT (Table 2); i.e., the $\Delta\Delta G_b$ values for each RT-NNRTI set were well compatible with the *in vitro* resistance data described above, suggesting that our modeling appropriately reflects the actual mode of binding between the RT molecule and the NNRTI.

In these models, EFV and NVP were predicted to bind to the hydrophobic pocket of RT, as demonstrated in the crystal structures (20, 26). In the wild-type RT, V106 and V179 contributed to the stabilization of the binding of EFV and NVP through hydrophobic interactions (Fig. 3A and C). However, the V106I and V179D mutations attenuated this stabilization by the following mechanisms. In the case of EFV, the V106I mutation caused a steric clash with the chlorine atom on one side of EFV and the V179D mutation caused electrostatic repulsion on the other side of EFV between the carbonyl oxygen atom of EFV and the carboxyl oxygen atoms of D179 in RT (Fig. 3B). In the case of NVP, the V106I mutation caused a steric clash with the aromatic ring of NVP, whereas the V179D mutation reduced hydrophobic contacts with NVP and the charged carboxyl atoms of D179 showed unfavorable contacts with the hydrophobic three-member ring of NVP (Fig. 3D). The effect of a single mutation on binding affinity was relatively moderate because a slight positional shift in EFV and NVP reduced unfavorable contacts. However, V106I V179D double mutations coincidentally caused repulsive interactions at the distinct sites of EFV and NVP which significantly attenuated the affinity of EFV and NVP for RT (Fig. 3B and D).

ETV has rotatable bonds that link aromatic rings. Therefore, it is conceivable that conformational plasticity allowed fine-tuning of ETV conformations for stable binding (Fig. 3E and F). In fact, superposition of the structural models of ETV-RT complexes showed that ETV changed its conformation and position depending on the mutations (Fig. 4A). In contrast, the conformations of EFV and NVP were more rigid due to a lack of rotatable bonds. Therefore, the conformations of EFV and NVP remained similar with various mutant RTs (Fig. 4B and C). These results suggest that the plasticity of the conformation of ETV plays a key role in maintaining its binding affinity for various mutant RTs, as reported in the crystal structural study (4).

DISCUSSION

The results of the present study indicated that the combination of two polymorphic mutations, V106I and V179D, alters the susceptibility of HIV-1 to EFV, as demonstrated in the resistance selection experiments (Fig. 1). Analysis of the recombinant monoclonal HIV-1 strains revealed that the combination confers significant resistance to EFV and NVP but

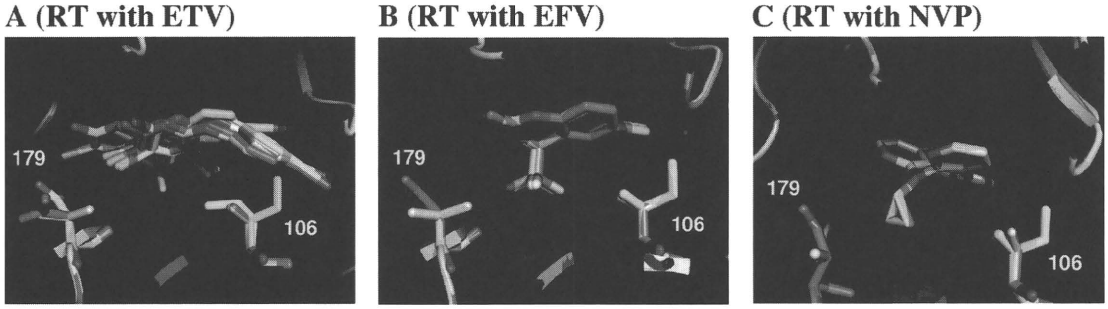

FIG. 4. Superposition of RT_{WT} and five mutant models. (A) RTs with ETV. (B) RTs with EFV. (C) RTs with NVP. NNRTIs and the 106th and 179th residues are highlighted by the stick configuration. White sticks, RT_{WT}; yellow, RT_{V106A}; green, RT_{V106I}; orange, RT_{V179D}; purple, $RT_{V106A/V179D}$; cyan, $RT_{V106I/V179D}$.

not to ETV, although each mutation alone could not alter NNRTI susceptibility (Table 2). Furthermore, one clinical HIV-1 isolate from a treatment-naïve patient who harbored V106I and V179D without any other resistance-associated mutation showed significant resistance to EFV and NVP but not to ETV (Table 3). In a previous study, Tee et al. (29) analyzed HIV-1 RT and protease sequences in 36 antiretroviral-treated patients with detectable viral loads but they could not find any known resistance-associated mutation in 8 patients. In one of their patients on EFV treatment (04MYKL1665), V106I and V179D coexisted in the HIV-1 RT according to GenBank (accession no. AY960901; accessed in October 2009). In a clinical trial of tipranavir, the HIV-1 isolate from one patient (case 48-1084), who experienced NNRTI-treatment failure, harbored V106I and V179D without any other NNRTI resistance-associated mutations (DQ880530) (2). These data strongly indicate that the combination of V106I and V179D also confers significant resistance to NNRTIs *in vivo*.

Structural modeling indicated that V106I and V179D cooperatively reduce NNRTI binding to EFV and NVP. ETV, however, exhibits structural plasticity and can avoid any disturbance caused by the combination of V106I and V179D. This specific structure probably contributes to the efficacy of ETV against many NNRTI-resistant HIV-1 strains, resulting in an excellent rate of response to ETV-containing salvage treatment (18, 21).

Both V106I and V179D are listed as minor ETV resistance-associated mutations in the current version of the IAS-USA Drug Resistance Mutation List (14), but both are not recognized as EFV and NVP resistance-associated mutations. They are often identified individually but rarely coexist in treatment-naïve individuals (Table 1). The combination of V106I and V179D, however, can be found in patients whose baseline HIV-1 held either V106I or V179D after failure of EFV- or NVP-containing treatment (2, 29). Considering that either V106I or V179D was identified in a significant portion of treatment-naïve patients (29/364; 8%) (Table 1), the above information on NNRTI resistance caused by the mutation combination should be recognized by all clinical specialists involved in the interpretation of genotype drug resistance tests and those physicians responsible for changing antiretroviral treatment regimens. In a previous study, we selected EFV-resistant HIV-1 by culture of monoclonal HIV-1 harboring another common polymorphic mutation, K103R ($HIV\text{-}1_{K103R}$), and found the additional emergence of V179D; we then confirmed that the combination of K103R and V179D conferred significant resistance to EFV and NVP (7). Considering these findings together, one assumes that the combinations of polymorphic mutations can reduce NNRTI susceptibility and that other combinations of polymorphic mutations can confer NNRTI resistance. Furthermore, mutations found to be important for one drug may actually have a greater effect on other drugs of the same class. Even polymorphic and minor resistance mutations should be considered carefully when interpreting the results of genotype testing.

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Long-Term Control of HIV-1 in Hemophiliacs Carrying Slow-Progressing Allele HLA-B*5101▽†

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HLA-B*51 alleles are reported to be associated with slow disease progression to AIDS, but the mechanism underlying this association is still unclear. In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome for Japanese hemophiliacs who had been infected with HIV-1 before 1985 and had been recruited in 1998 for this study. HLA-B*5101$^+$ hemophiliacs exhibited significantly slow progression. The analysis of HLA-B*5101-restricted HIV-1-specific cytotoxic T-lymphocyte (CTL) responses to 4 HLA-B*-restricted epitopes in 10 antiretroviral-therapy (ART)-free HLA-B*5101$^+$ hemophiliacs showed that the frequency of Pol283-8-specific CD8$^+$ T cells was inversely correlated with the viral load, whereas the frequencies of CD8$^+$ T cells specific for 3 other epitopes were positively correlated with the viral load. The HLA-B*5101$^+$ hemophiliacs whose HIV-1 replication had been controlled for approximately 25 years had HIV-1 possessing the wild-type Pol283-8 sequence or the Pol283-8V mutant, which does not critically affect T-cell recognition, whereas other HLA-B*5101$^+$ hemophiliacs had HIV-1 with escape mutations in this epitope. The results suggest that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs is associated with a Pol283-8-specific CD8$^+$ T-cell response and that lack of control of HIV-1 is associated with the appearance of Pol283-8-specific escape mutants.

Human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells play a critical role in the control of HIV-1 infections (26, 5), but HIV-1 escape occurs during acute and chronic phases of an HIV-1 infection (6, 14). There are several mechanisms affording HIV-1 escape from the host immune system. They include the appearance of mutants that escape from HIV-1-specific cytotoxic T lymphocytes (CTLs) (6, 14) and neutralizing antibodies (27, 47, 48), impaired recognition of HIV-1-infected cells by HIV-1-specific CTLs due to Nef-mediated downregulation of HLA class I molecules (8, 42), and impaired function of HIV-1-specific T cells (3).

It is well known that long-term nonprogressors (LTNPs), who remain disease free and have very low or undetectable viral loads (VLs) in the absence of antiretroviral therapy (ART), exist as a very small population of HIV-1-infected individuals (7, 21, 38). A small minority of these LTNPs were infected by HIV-1 containing deletions in viral accessory molecules (10, 17, 24). HLA alleles such as HLA-B*57/5801, HLA-B*27, and HLA-B*51 are associated with slow progression to AIDS (19, 22, 37). Indeed, it is reported that many LTNPs carry these HLA alleles (31, 36). These findings imply that HIV-1-specific CTLs restricted by these alleles may play an important role in the control of HIV-1 replication in LTNPs. The mechanism of control of HIV-1 replication has been analyzed in LTNPs and slow progressors carrying HLA-B*57/5801, HLA-B*27, or HLA-B*13, and has been related to the Gag-specific CD8$^+$ T-cell epitopes presented by these alleles (9, 11, 14, 16, 34). On the other hand, the mechanism underlying the association between HLA-B*5101 and slow progression remains unclear. To date, no study of the mechanism of control of HIV-1 in HLA-B*5101$^+$ LTNPs has been reported.

Since the data indicate that HIV-1 replication can be controlled for more than 20 years in LTNP hemophiliacs, analysis of HIV-1-specific immune responses and HIV-1 in these patients is useful for investigating the immunological control of HIV-1. In Japan, HLA-B*57/58 and HLA-B*27 are very rare alleles (18). Therefore, it was speculated that only HLA-B*51 would play an important role in the control of HIV-1 replication in HIV-1-infected Japanese donors.

We showed previously that 2 Pol peptides and 1 Gag peptide were HLA-B*5101-restricted immunodominant CTL epitopes (45). Two Pol-specific CTLs are known to have strong abilities to suppress HIV-1 replication *in vitro* (43). Our recent study using 9 cohorts showed that of these T cells, Pol283-specific CTLs select mutations at position 8 (position 135 of reverse transcriptase [RT]) in the epitope (20). A Thr mutation at position 8 (8T) was found predominantly in HIV-1-infected HLA-B*5101$^+$ donors, whereas the 8R, 8L, and 8V mutations were also found in these donors. The 8T, 8L, and 8R mutants had fitness similar to

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that of the wild-type virus, whereas the 8V mutation had a higher fitness cost than the others.

In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome in Japanese hemophiliacs infected with HIV-1. In addition, we investigated the role of HLA-B*5101-restricted HIV-1-specific CTLs *in vivo* in HLA-B*5101$^+$ LTNP and slow-progressing Japanese hemophiliacs who had not been treated with antiretroviral therapy for approximately 25 years. Our results revealed a role for Pol283-8-specific HLA-B*5101-restricted HIV-1-specific CTLs in the long-lasting (approximately 25 years) control of HIV-1 replication.

MATERIALS AND METHODS

Patients. One hundred eight Japanese hemophiliacs who had been infected with HIV-1 before 1985, mostly around 1983, were recruited for the present study, which was approved by the ethics committees of Kumamoto University and the National Center for Global Health and Medicine. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Patient HLA type was determined by standard sequence-based genotyping. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood.

Cells. C1R and 721.221 cells expressing HLA-B*5101 (C1R-B*5101 and 721.221-B5101, respectively) were generated previously (15, 33, 44). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

HIV-1 clones. An infectious proviral clone of HIV-1, pNL-432, and its mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously (1). Pol283-8 and Pol743-9 mutant (Pol283-8L, -8T, -8V, and -8R; Pol743-1I, -5I, and -4I5I) viruses were generated based on pNL-432 by using the GeneTailor site-directed mutagenesis system (Invitrogen).

HLA class I tetramers. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as described previously (2). Four HIV-1 specific epitopes (Pol283-8, Pol743-9, Gag327-8, and Rev71-11) (45) were used for the refolding of HLA-B*5101 molecules. Phycoerythrin (PE)-labeled streptavidin (Molecular Probes) was used for the generation of the tetramers.

Flow cytometric analysis using tetramers. PBMCs were incubated with the tetramers at 37°C for 30 min. The cells were subsequently washed twice with RPMI–10% newborn calf serum (NCS) and were then stained with an anti-CD8 monoclonal antibody (MAb). Next, they were incubated at 4°C for 30 min and were then washed twice with RPMI–10% NCS. The cells were finally resuspended in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, and then the percentage of tetramer-positive cells among the CD8$^+$ population was determined by using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA).

Generation of CTL clones. Pol283-8-specific CTL clones and Pol743-9-specific CTL clones were generated from HIV-1-specific bulk-cultured T cells by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) containing 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-B*5101 cells prepulsed with the corresponding peptide at 1 μM in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human recombinant interleukin-2 [rIL-2]) (43).

CTL assay for target cells infected with HIV-1. The cytotoxicity of CTL clones for 721.221-B5101 cells infected with HIV-1 (>30% p24 antigen [Ag]-positive cells) was determined by the standard ^{51}Cr release assay as described previously (42). The infected cells were incubated with 150 μCi $Na_2{}^{51}CrO_4$ in saline for 60 min, and then the infected cells were washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark) with effector cells at an effector-to-target cell (E:T) ratio of 2:1. The cells were then incubated for 6 h at 37°C. The supernatants were collected and analyzed with a gamma counter.

Assay for suppression of HIV-1 replication by HIV-1-specific CTLs. The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (42). CD4$^+$ T cells isolated from PBMCs were derived from an HIV-1-seronegative individual with HLA-B*5101. After the CD4$^+$ T cells had been incubated with the desired HIV-1 clones for 4 h at 37°C, they were washed three times with R10 medium. The HIV-1-infected CD4$^+$ T cells were then cocultured with HIV-1-specific CTL clones. From day 3 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) (HIV-1 p24 Ag ELISA kit; ZeptoMetrix).

Sequencing of proviral DNA or plasma RNA. Genomic DNA was extracted from PBMCs by using a QIAamp DNA blood minikit (Qiagen). Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp Mini Elute virus spin kit (Qiagen). cDNA was synthesized from the RNA with SuperScript II and random primers (Invitrogen). We amplified HIV RT and integrase sequences by nested PCR using RT-specific primers 5′-CCAAAAGT TAAGCAATGGCC-3′ and 5′-CCCATCCAAAGGAATGGAGG-3′ or 5′-CC TTGCCCCTGCTTCTGTAT-3′ for the first round of PCR and 5′-AGTTAGG AATACCACACCCC-3′ and 5′-GTAAATCCCCACCTCAACAG-3′ or 5′-AA TCCCCACCTCAACAGAAG-3′ for the second round and integrase-specific primers 5′-ATCTAGCTTTGCAGGATTCGGG-3′ and 5′-CCTTAACCGTAG TACTGGTG-3′ or 5′-CCTGATCTCTTACCTGTCC-3′ for the first round of PCR and 5′-AAAGGTCTACCTGGCATGGG-3′ or 5′-TTGGAGAGCAATG GCTAGTG-3′ and 5′-AGTCTACTTGTCCATGCATGGC-3′ for the second round. PCR products were either sequenced directly or cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems), and sequences were analyzed by use of an ABI PRISM 310 genetic analyzer.

Cell surface staining and intracellular cytokine staining (ICC assay). PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12 to 14 days. These cultured PBMCs were assessed for gamma interferon (IFN-γ)-producing activity as previously described (42). After C1R-B*5101 cells had been incubated for 60 min with epitope peptides (1 μM), they were washed twice with RPMI 1640 containing 10% FCS. These C1R cells and the cultured PBMCs were incubated at 37°C for 6 h at an effector-to-stimulator ratio of 2:1 or 4:1 after the addition of brefeldin A (10 μg/ml). Next, the cells were stained with an anti-CD8 MAb (Dako Corporation, Glostrup, Denmark), fixed with 4% paraformaldehyde at 4°C for 20 min, and then permeabilized at 4°C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and were then stained with an anti-IFN-γ MAb (BD Bioscience Pharmingen, San Diego, CA). Finally, they were resuspended in PBS containing 2% paraformaldehyde, and then the percentage of CD8$^+$ cells positive for intracellular IFN-γ was determined by using a FACSCalibur flow cytometer.

RESULTS

Association of HLA-B*5101 with long-term control of HIV-1 in HIV-1-infected Japanese hemophiliacs. We recruited 108 Japanese hemophiliacs who had been infected with HIV-1 before 1985. Eighteen of the patients had not been treated with any antiretroviral therapy (ART) and had CD4 counts of >350 (very-slow-progressor [VSP] group) by 1998, whereas the other 90 patients had been treated with ART and/or had a CD4 count of <350 (slow-progressor [SP] group). The frequency of HLA-B*5101 in the VSP group (9 of 18 donors [50.0%]) was higher than that in the SP group (15 of 90 donors [16.7%]), and the difference between these 2 groups was significant (P, 0.01). We analyzed the association of HLA class I alleles with disease progression during the years 1998 to 2007 in the VSP group. The 9 HLA-B*5101$^+$ VSP hemophiliacs exhibited significantly slower progression of the disease over this period than the 9 HLA-B*5101$^-$ subjects (Fig. 1), and no other HLA-B alleles or HLA-A/DR alleles showed any significant influence on the progression of the disease in this group (not shown). One HLA-B*3501$^+$ VSP hemophiliac was found in the HLA-B*5101$^+$ group, but none were found in the HLA-B*5101$^-$ group, indicating that HLA-B*3501, which is associated with rapid progression to AIDS, did not affect the results for the 2 VSP groups. Other HLA-A/B/DR alleles were not associated with the HLA-B*5101$^+$ or the HLA-B*5101$^-$ group (see Table S1 in the supplemental material). These results, taken together, show that the HLA-B*5101 allele was

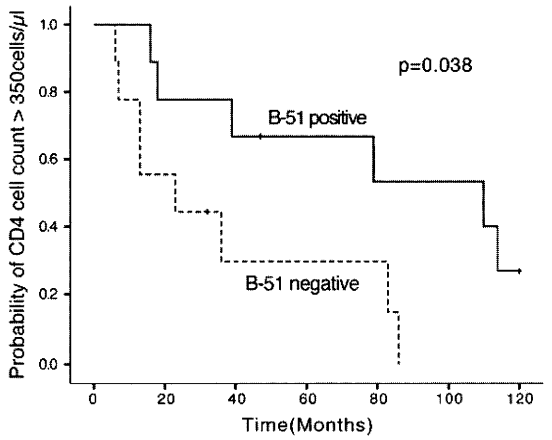

FIG. 1. Association of HLA-B*5101 with slow progression to AIDS. Kaplan-Meier survival analysis was used to estimate the time to the first CD4 cell count (24-week time-weighted average levels of CD4 cells) of $<350/\mu l^3$ for 9 HLA-B*5101-positive (solid line) and 9 HLA-B*5101-negative (dashed line) hemophiliacs who had not been treated with antiretroviral therapy (ART) and who had a CD4 count of $>350/\mu l$ in 1998.

still associated with slow progression of the disease more than 20 years postinfection.

Control of HIV-1 replication by HLA-B*5101-restricted CD8$^+$ T cells. A previous study demonstrated that 2 types of HLA-B*5101-restricted CTLs, Pol283-8 (TAFTIPSI)-specific and Pol743-9 (LPPVVAKEI)-specific CTLs, suppressed HIV-1 replication *in vitro* much more strongly than did other HLA-B*5101-restricted CTLs (43), suggesting that these CTLs may play a key role in the control of HIV-1 in the HLA-B*5101$^+$ SP group. To investigate the control of HIV-1 by these CTLs, we selected 10 HLA-B*5101-positive donors (8 VSPs and 2 SPs) who had not been treated with ART by 1998 and whose PBMC samples were available for analysis of HLA-B*5101-restricted CTLs (see Fig. S1 and Table S2 in the supplemental material). Three of the 8 VSP patients had VLs below 1,000 copies at all time points tested and were classified as LTNPs. We found that only 3 of the 108 HIV-1-infected hemophiliacs (KI-021, KI-051, and KI-124) were LTNPs for approximately 25 years and that all 3 of these LTNPs carried HLA-B*5101. We generated 4 HLA-B*5101 tetramers carrying Pol283-8, Pol743-9, Gag327-9, or Rev71-11, and we used them to determine the frequencies of HIV-1-specific CD8$^+$ T cells among PBMCs from these 3 LTNPs (Table 1 and Fig. 2). KI-021 had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells but neither Gag327-9- nor Rev71-11-specific CD8$^+$ T cells during the years 1997 to 2005 (Fig. 2A). KI-051 also had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells, whereas this patient had no Rev71-11-specific CD8$^+$ T cells and a low number of Gag327-9-specific CD8$^+$ T cells during the years 1999 to 2005 (Fig. 2B). KI-124 had Pol283-8-, Pol743-9-, and Gag327-9-specific CD8$^+$ T cells (Table 1). These results suggest that the 2 Pol-specific CD8$^+$ T cells may play an important role in the control of HIV-1 in these LTNPs carrying HLA-B*5101.

Selection of escape mutations of the Pol283-8 epitope in very slow progressors. Of the 8 HLA-B*5101$^+$ VSP hemophiliacs, KI-127 had Pol283-8-specific CD8$^+$ T cells at a low frequency in 1998, when the plasma viral load (pVL) was very low, whereas later this patient lost the response, and the pVL increased from an undetectable level to more than 10^3 copies (Fig. 2C). The other 4 VSPs, excluding 3 LTNBs, either had a low number of Pol283-8-specific CD8$^+$ T cells or did not have any of these cells at any time points studied. These results suggest that Pol283-8-specific CD8$^+$ T cells rather than Pol743-9-specific CD8$^+$ T cells may control HIV-1 *in vivo*.

To clarify the role of these HLA-B*5101-restricted CD8$^+$ T cells in the control of HIV-1 *in vivo*, we analyzed the correlation between the frequency of the HLA-B*5101-restricted CD8$^+$ T cells and the pVL in 10 HLA-B*5101$^+$ hemophiliacs. The frequency of Pol283-8-specific CD8$^+$ T cells was negatively correlated with the pVL (P, 5.6×10^{-8}), whereas the frequency of the other T cells was positively correlated with the pVL (Fig. 3). These results support the idea that Pol283-8-specific CD8$^+$ T cells drive the suppression of HIV-1 replication *in vivo*.

We speculated, therefore, that escape mutants within Pol283-8 epitopes were selected in slow progressors over a 25-year period, because these epitope-specific CTLs are thought to provide strong immune pressure on HIV-1. Two of the LTNPs had the Pol283-8V mutant, whereas the third had wild-type Pol283 in July 2002 but the 8V mutant in October

TABLE 1. Numbers of 4 types of HLA-B*5101-restricted CD8$^+$ T cells among HLA-B*5101$^+$ HIV-1-infected hemophiliacs

Patient	Median VL (copies/ml)[a]	Median no. of CD4 cells/μl[a]	Median no. (frequency) of HLA-B*5101-restricted CD8$^+$ T cells[b]				No. of times PBMCs were tested (dates)[c]
			Pol743	Pol283	Gag327	Rev71	
KI-021	50	618	1,910 (0.39)	1,900 (0.40)	<100 (0)	<100 (0)	10 (8/1997–11/2005)
KI-051	50	737	3,222 (0.53)	5,186 (0.87)	1,082 (0.16)	<100 (0)	5 (10/1999–9/2005)
KI-124	570	850	3,126 (0.43)	1,745 (0.24)	1,381 (0.19)	<100 (0)	8/2001
KI-386	360	459	3,164 (0.40)	554 (0.07)	5,774 (0.73)	396 (0.05)	8/2006
KI-363	1,700	676	6,696 (0.54)	1,488 (0.12)	496 (0.04)	1,116 (0.09)	11/1998
KI-127	5,500	597	8,100 (0.79)	257 (0.02)	23,411 (2.33)	<100 (0.01)	9 (2/1998–4/2006)
KI-121	16,650	327	4,853 (0.59)	134 (0.02)	<100 (0)	395 (0.04)	2 (12/1999, 8/2001)
KI-032	25,500	226	9,153 (1.80)	<100 (0)	344 (0.09)	<100 (0)	2 (10/2002, 9/2005)
KI-007	39,500	387	1,084 (0.12)	394 (0.05)	6,278 (0.68)	1,029 (0.12)	2 (6/2001, 4/2002)
KI-026	40,000	526	10,705 (1.32)	<100 (0)	6,164 (0.76)	568 (0.07)	7/2005

[a] At the time of tetramer analysis.
[b] Median number of HLA-B*5101-restricted CD8$^+$ T cells/μl among PBMCs (median frequency of HLA-B*5101-restricted T cells among CD8$^+$ T cells [expressed as a percentage]).
[c] If PBMCs were tested only once, only the date (month/year) is given.

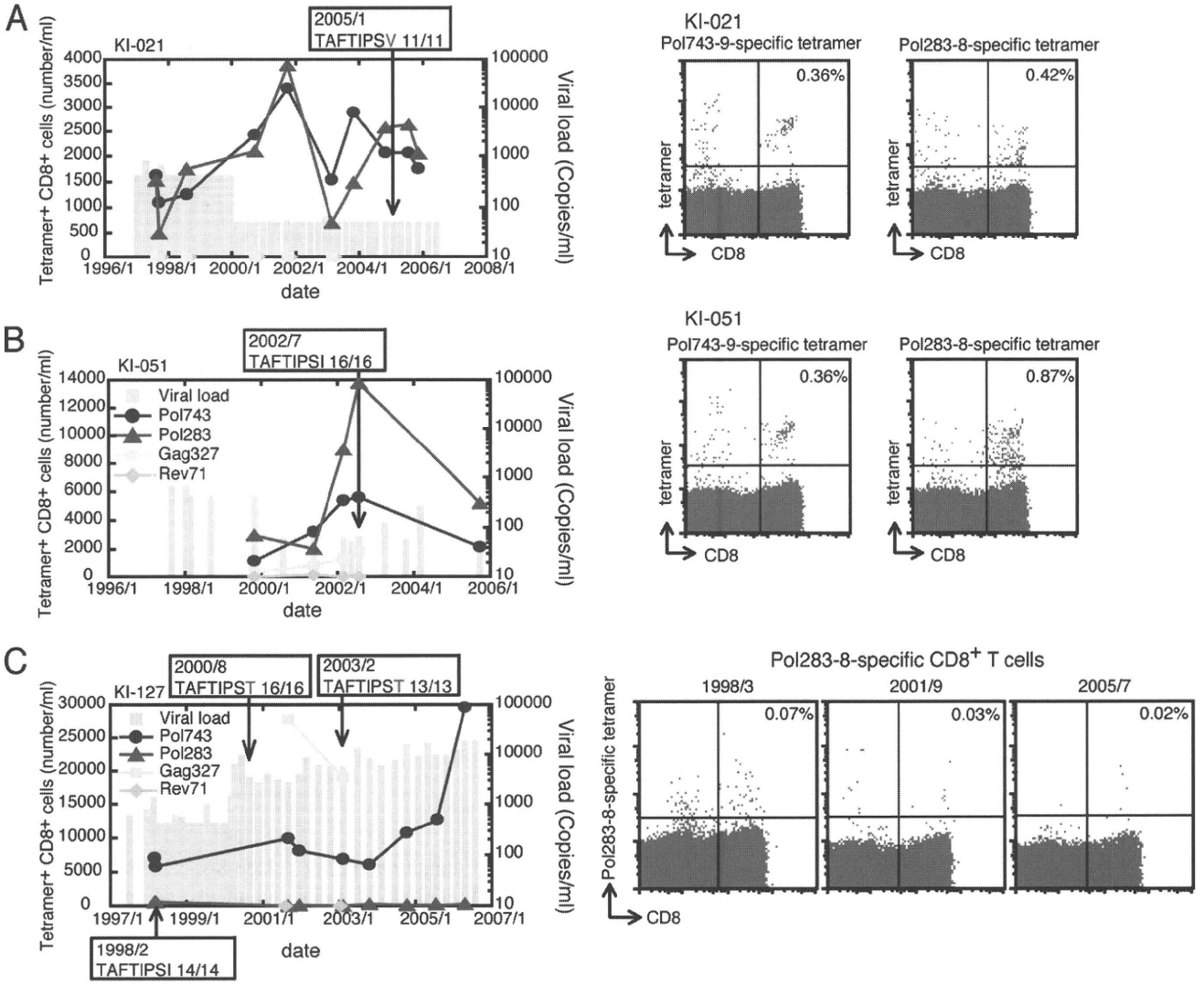

FIG. 2. Longitudinal analysis of HLA-B*5101-restricted $CD8^+$ T cells and Pol283 epitope sequences in 3 slow-progressing hemophiliacs. Four types of HIV-1-specific $CD8^+$ T cells were detected by use of specific tetramers. PBMCs from KI-021 (A), KI-051 (B), and KI-127 (C) were analyzed by using Pol743-9-specific and Pol283-8-specific tetramers. The percentage of tetramer-positive cells among the $CD8^+$ T-cell population is given in the upper right quadrant of each histogram. The sequence of the Pol283-8 epitope from each patient is shown. The detection limit of pVL was 400 copies/ml until 2000 and 50 copies/ml after 2000.

2006 (Table 2). As previously noted (34), Pol283-8-specific CTL clones showed the same killing activity toward target cells prepulsed with the Pol283-8V peptide as toward those prepulsed with the wild-type peptide. These T cells revealed similar killing activity toward 721.221-B*5101 cells infected with NL-432 carrying Pol283-8V (NL-Pol283-8V) as toward those infected with NL-432 (see Fig. S2A in the supplemental material) and only a marginally weaker ability to suppress the replication of NL-Pol283-8V (see Fig. S2B in the supplemental material). In contrast, the 5 VSPs and 2 SPs had Pol283-8T or Pol283-8R mutants (Table 2). Three Pol283-8-specific CTL clones failed to kill target cells infected with NL-432 carrying these mutants (NL-Pol283-8T and NL-Pol283-8R [see Fig. S2A in the supplemental material]) or to suppress the replication of these mutants (see Fig. S2B in the supplemental material), indicating that these were escape mutants.

Longitudinal analysis of KI-127 showed that the 8T mutant appeared in August 2000, when the VL had increased approximately 10-fold, whereas wild-type Pol283 was found in February 1998, when the VL was very low or undetectable (Fig. 2C). Previous population analysis using 9 cohorts showed strong association between HLA-B*51 and Pol283-8T (20). These observations together suggest that the 8T mutant is an escape mutant selected by Pol283-specific CTLs and implies that escape from this epitope reduces immune control of HIV-1.

***In vitro* selection of Pol283 escape mutants by Pol283-specific CTLs.** The results shown in Fig. 4 suggested that Pol283-specific CTLs selected 8T, 8R, and 8L escape mutants. To further confirm the selection of these mutants by Pol283-specific CTLs, we investigated whether Pol283-specific CTLs selected these mutant viruses *in vitro* when the CTLs were cul-

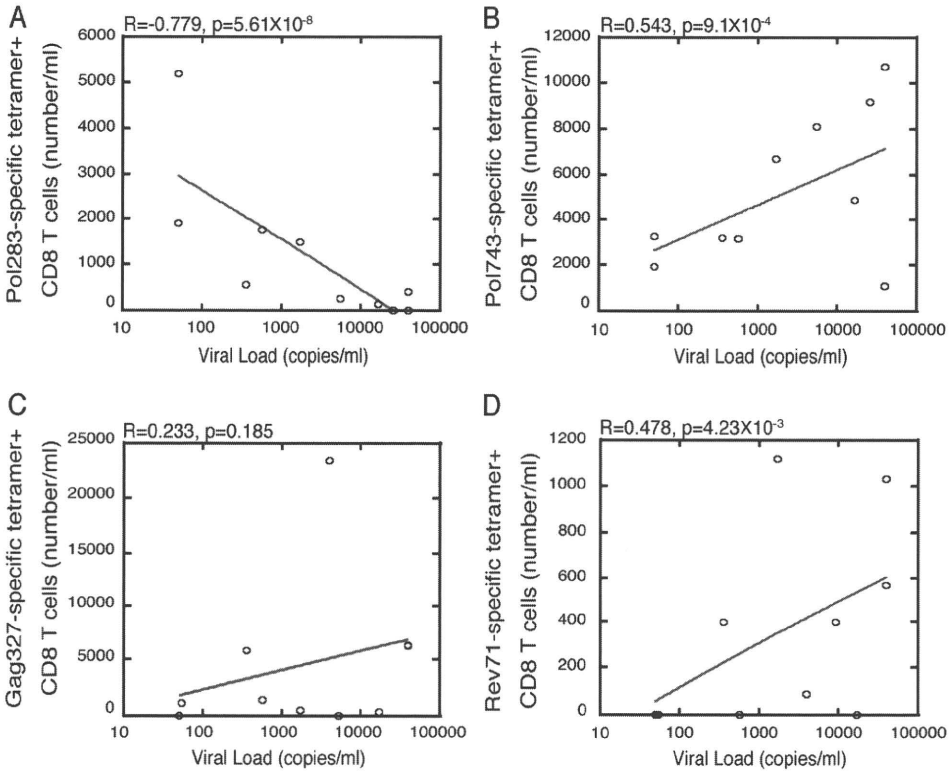

FIG. 3. Correlation of the number of HLA-B*5101-restricted CD8+ T cells with the viral load. The number of Pol283-8-specific (A), Pol743-9-specific (B), Gag327-specific (C), or Rev71-specific (D) CD8+ T cells among PBMCs from 10 HLA-B*5101+ hemophiliacs was measured at 1 time point or at 2 to 10 different time points (see Table 1) by using specific tetramers. The correlation of the median number of tetramer-positive cells with the median viral load was analyzed.

tured with HLA-B*5101-positive CD4+ T cells infected with NL-432 and the mutant virus together. Pol283-specific CTL clones selected these 3 mutant (8T, 8R, and 8L) viruses rapidly in this assay (Fig. 4A to C), supporting the notion that these mutants were selected as escape mutants by Pol283-specific CTLs.

Long-term maintenance of Pol283-8-specific memory CD8+ T cells and failure of induction of escape mutant-specific CD8+ T cells. If the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with HIV-1 carrying the Pol283-8 wild-type epitope, we can speculate that the donors had Pol283-8-specific memory CD8+ T cells but failed to elicit

TABLE 2. Sequences of Pol283-8 and Pol743-9 epitopes in HLA-B*5101+ HIV-1-infected hemophiliacs

Patient	Epitope				VL (copies/ml)	Date (mo/yr) of PBMC testing[b]
	Pol283-8		Pol743-9			
	Sequence	Clonal frequency[a]	Sequence	Clonal frequency		
NA[c] (wild-type sequence)	TAFTIPSI		LPPVVAKEI			
KI-021	-------V	11/11	---------	10/12	<50	1/2005
KI-051	--------	16/16	---------	15/15	63	7/2002
	-------V	DS	ND[d]	ND	<50	10/2006
KI-124	-------V	14/14	---------	14/15	600	8/2001
KI-386	-------T	DS	---------	DS	1,200	10/2006
KI-363	-------T	DS	---------	DS	1,700	11/1998
KI-127	-------T	13/13	---------	17/17	5,300	2/2003
KI-121	-------T	16/16	I--------	12/13	9,300	12/1999
KI-032	-------T	13/13	---------	15/15	17,000	10/2002
KI-007	-------R	15/16	---II----	18/18	33,000	6/2001
KI-026	-------T	DS	I--------	DS	28,000	1/2004

[a] Expressed as (number of clones carrying the indicated sequence)/(number of clones tested). DS, direct sequence.
[b] The sequence for patient KI-021 is from proviral DNA; those for all other patients are from plasma RNA.
[c] NA, not applicable.
[d] ND, not determined.

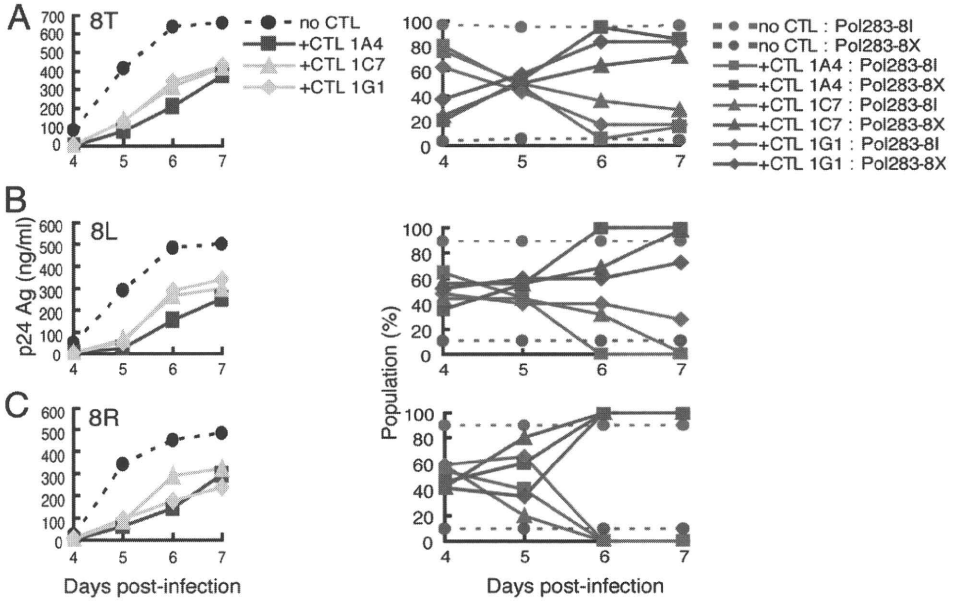

FIG. 4. *In vitro* selection of Pol283 escape mutants by a Pol283-8-specific CTL clone. T1 cells were infected with paired viruses (NL-432 [Pol283-8I] and a mutant virus [Pol283-8L, -8T, or -8R]) at a ratio of 9:1. The infected cells were incubated with Pol283-8-specific CTL clones at an E:T ratio of 1:0.05. The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram. From day 4 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in these supernatants was measured by an ELISA. The data obtained by using the mixture of Pol283-8T, -8L, or -8R with Pol283-8I are shown in panels A, B, and C, respectively.

Pol283-8T-specific $CD8^+$ T cells after the Pol283-8T mutation appeared. None of 4 HLA-B*5101^+ hemophiliac donors carrying Pol283-8T (KI-032, KI-121, and KI-127 [Table 2] and 1 ART-treated hemophilic donor, KI-078 [data not shown]) had detectable Pol283-8-specific $CD8^+$ T cells by analysis using the specific tetramers. But they may have had very small numbers of memory $CD8^+$ T cells. To induce Pol283-8-specific $CD8^+$ T cells from a possible Pol283-8-specific memory T-cell source, we stimulated PBMCs from these patients with the Pol283-8 peptide and then measured the number of Pol283-8-specific $CD8^+$ T cells in 2-week cultures. The KI-127 and KI-078 cultures indeed showed the presence of Pol283-8-specific $CD8^+$ T cells, but KI-127 lost the detectable memory response by April 2006 (Fig. 5), indicating that these 2 patients could maintain Pol283-8-specific memory $CD8^+$ T cells for more than 20 years. In contrast, Pol283-8T-specific $CD8^+$ T cells were not detected among PBMCs from any of these 4 donors after 2 weeks in culture (Fig. 5), indicating that the Pol283-8T escape mutant did not elicit specific $CD8^+$ T cells *in vivo*. These results support the idea that the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with the wild-type virus. Similarly, Pol283-8R-specific $CD8^+$ T cells were not detected in KI-007, although this patient had Pol283-8-specific memory $CD8^+$ T cells (Fig. 5), supporting the notion that the 8R mutant was an escape mutant selected by Pol283-8-specific CTLs and failed to elicit these escape mutant-specific CTLs.

DISCUSSION

It is well known that HLA-B*57 and -B*27 are associated with slow progression to AIDS (19, 37). HLA-B*57-mediated and HLA-B*27-mediated effects on disease progression are seen early and late, respectively, during an infection (6, 14). In the present study, we analyzed 108 HIV-1-infected Japanese hemophiliacs. In Japan, 1,439 patients had been infected with HIV-1 before 1985, mostly around 1983. At present, only 801 of these patients remain alive. Since they had not been treated with highly active antiretroviral therapy (HAART) before 1997, the survivors would seem to be slow progressors. This cohort does not include a large number of patients, because it is not easy to recruit a large number of HIV-1-infected hemophiliacs in Japan, where only 800 are still alive. We found that HLA-B*5101 had effects on the slow progression of the disease in the late phase (both in 1998 and during the years from 1998 to 2007), even when a small number of samples was analyzed. Our recent study also revealed that HLA-B*5101^+ hemophiliacs had lower VLs and higher CD4 counts than HLA-B*5101^- hemophiliacs but that only the CD4 count was significantly higher in HLA-B*5101^+ than in HLA-B*5101^- hemophiliacs (20). These findings support the idea that HLA-B*5101-restricted immune responses are associated with slow progression to AIDS.

Pol283-8, Pol743-9, and Gag327-9 are thought to be immunodominant HIV-1 epitopes, because CTLs specific for them were frequently detected in chronically HIV-1 infected HLA-B*5101^+ individuals (45). A previous study demonstrated that Pol283-8-specific and Pol743-9-specific CTLs suppress HIV-1 replication strongly but that Gag327-9-specific CTLs suppress it only weakly *in vitro* (43), suggesting that HIV-1 replication can be suppressed *in vivo* by Pol283-8-specific and Pol743-9-specific CTLs. In the present study, we demonstrated that a higher number of Pol283-8-specific $CD8^+$ T cells was detected predominantly in LTNPs, whereas Pol743-9-specific $CD8^+$ T cells were found at higher levels in all 10 of the SP hemophiliac

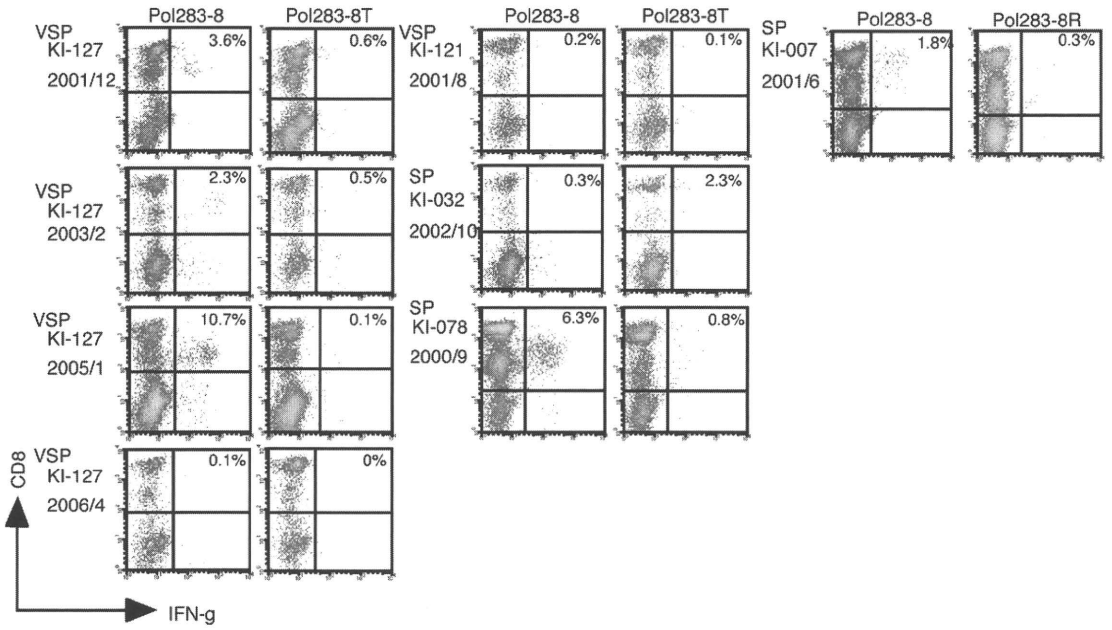

FIG. 5. Induction of Pol283-8-specific CD8+ T cells from PBMCs of 2 very slow progressors and 3 slow progressors. PBMCs from 2 very slow progressors (KI-127 and KI-121) and from 3 slow progressors (KI-032, KI-007, and KI-078) were stimulated with the Pol283-8 epitope peptide or the Pol283-8T or -8R peptide and were then cultured for 12 to 14 days. The cultured cells were stimulated with C1R-B*5101 cells prepulsed with the peptide. IFN-γ-producing CD8+ T cells were measured by using flow cytometry. The percentages of IFN-γ-producing CD8+ T cells are given in the upper right quadrants.

patients examined. ART-treated HLA-B*5101+ patients also carried Pol743-9-specific CD8+ T cells but not Pol283-8-specific CD8+ T cells (data not shown). The frequency of Pol283-specific CD8+ T cells was negatively correlated with the pVL, whereas the frequencies of the other 3 types of T cells were positively correlated with the pVL (Fig. 3). The longitudinal analysis of KI-127 showed that the VL increased after the 8T mutant appeared. This suggests that Pol283-specific CTLs may control HIV-1 in this patient, but the possibility that other CTLs also control HIV-1 cannot be excluded. These results support the notion that Pol283-8-specific CTLs play a key role in the control of HIV-1 in chronically HIV-1 infected HLA-B*5101+ hemophiliacs.

Previous studies showed that Gag-specific responses are negatively correlated with VL in chronically HIV-1 infected individuals (23, 25, 28, 49). Especially HLA-B*57/5801-, HLA-B*27-, HLA-B*13-, or HLA-B*63-restricted Gag-specific CD8+ T-cell responses are related to a low viral load (12, 16, 23, 34, 49). However, these studies had been performed with Caucasian and African cohorts. Since HLA-B*57/5801, HLA-B*27, and HLA-B*13 are very rare in Japan, Gag-specific CD8+ T-cell responses might not be related to a low pVL in Japanese patients. For the HLA-B*5101+ hemophiliacs studied here, it is striking that Pol283-specific CD8+ T-cell responses were much more effective in the control of HIV replication than Gag327-specific CD8+ T-cell responses. A previous study revealed that simian immunodeficiency virus (SIV)-infected cells are recognized earlier by Pol-specific T cells than by Nef-specific T cells (39). These results suggest that Pol-specific responses may be important in the control of HIV-1, and not only in the Japanese population. This is potentially an important result in relation to vaccine design and the specificity of the CD8+ T-cell responses that must be induced to achieve immune control of HIV.

Our recent study using 9 cohorts showed that there are 4 mutations (8T, 8R, 8L, and 8V) at position 8 of the Pol283 epitope, that the frequency of the 8T variant is significantly higher in HLA-B*5101+ donors than in HLA-B*5101− donors, and that some acutely infected HLA-B*5101+ subjects who had been infected with the wild-type virus had the 8T virus at only 6 or 12 months after the first test (20), indicating that the 8T mutant is selected by Pol283-specific CTLs. In the present study, we revealed that the Pol283-8T escape mutation was detected for the first time approximately 20 years post-HIV-1 infection in KI-127, indicating that this mutation had been slowly selected by Pol283-8-specific CTLs in this donor. Pol283-8R and Pol283-8L were also apparently escape mutants, because Pol283-8-specific CTLs failed to suppress the replication of HIV-1 carrying these mutants. However, the frequency of these mutations is not significantly higher in HLA-B*5101+ donors than in HLA-B*5101− donors (20), suggesting that other, non-HLA-B*5101-restricted CTLs may also select these particular mutants. Nonetheless, it is clear that the HLA-B*5101-restricted Pol283-specific CTLs select the 8R mutant, because KI-007, who had the 8R mutant virus, possessed Pol283-specific memory T cells (Fig. 5), and one HLA-B*5101+ subject with an acute HIV infection who had been infected with the wild-type virus had the 8R mutant 12 months after the first test (20).

The Pol283-8V mutant was found in only 6 of 60 HLA-B*5101+ donors, including 3 LTNP hemophiliacs (data not shown). Of the 3 nonhemophiliacs, 2 were progressors and 1 was a slow progressor. Since this mutation is rare and it is speculated that the mutations had not accumulated 25 years

ago, it is unlikely that the 3 LTNP hemophiliacs had been infected with this mutant virus. On the other hand, the 3 nonhemophiliacs may have been infected with the 8V mutant. The 8V mutation did not influence the killing activity of Pol283-8-specific CTLs toward target cells infected with the HIV-1 mutant, whereas the ability of CTLs to suppress replication was significantly weaker for the Pol283-8V mutant than for the wild-type virus. Previous studies showed that HIV-1-specific CTL clones can partially suppress HIV-1 replication but fail to kill HIV-1-infected $CD4^+$ T cells (42, 45), indicating that the replication suppression assay is more sensitive than the CTL assay. Since Pol283-8-specific CTLs cannot completely suppress the replication of the 8V mutant virus, and since the 8V virus has a higher fitness cost than the wild-type virus, the donors selecting this mutant virus can be LTNP hemophiliacs. However, it still remains unclear why the 8V virus appears in both LTNPs and progressors. We are now analyzing the HLA-B^*5101^+ nonhemophiliacs carrying the 8V mutants in order to compare them with the LTNPs carrying the 8V mutant.

Our previous study on the crystal structure of the HLA-B^*5101–Pol283-8 peptide complex showed that the C-terminal anchor (PC) pocket is hydrophobic and relatively small compared with those of the serologically close alleles, HLA-B^*3501 and -B^*5301, whose C-terminal preferential amino acids include aromatic amino acids (30). Those findings explain why the PC residues for HLA-B^*5101 are preferably aliphatic amino acids and not bulky aromatic amino acids. The PC residue is tethered with well-ordered polar and hydrophobic interactions, as observed in other major histocompatibility complex (MHC) class I molecules (Fig. 6A). Thus, the amino acid substitutions of the PC residue did not likely lead to large rearrangements of this network, and so the orientations of the side chains were presumably maintained. In the case of the 8R mutation, the PC pocket was not large enough to accommodate the Arg residue (Fig. 6B), conferring structural changes around the PC pocket that could possibly result in a lack of binding activity toward HLA-B^*5101 (2). The 8L mutant exhibited slightly reduced binding activity toward HLA-B^*5101 and CTL recognition for 8L peptide-pulsed target cells but no CTL response to 8L mutant-infected cells, suggesting that the mutation had a deleterious effect on antigen presentation in the system for export to the cell surface. The 8V mutation would delete only one methylene group from the Ile residue and thus would presumably have only a small influence on the binding to HLA-B^*5101 as well as on its specific T-cell receptor (TCR) recognition. On the other hand, the Pol283-8T mutation likely introduces a hydrophilic OH group that probably is not appropriate for the hydrophobic pocket, resulting in diminished binding activity (43). Furthermore, the Pol283-8T mutation was detrimental to the CTL response and thus may also have induced a structural rearrangement that had a negative effect on TCR recognition.

A higher accumulation of Pol283-8 escape mutations is found in the Japanese population than in other populations, because the frequency of HLA-B^*51 is much higher in Japan than in other countries (20). The fitness of the 8T, 8R, and 8L viruses is similar to that of the wild-type virus, and these escape mutants do not revert to wild-type viruses in HLA-B^*5101^- donors (20). The donors with escape mutant viruses failed to elicit escape mutant-specific CTLs. These findings suggest a

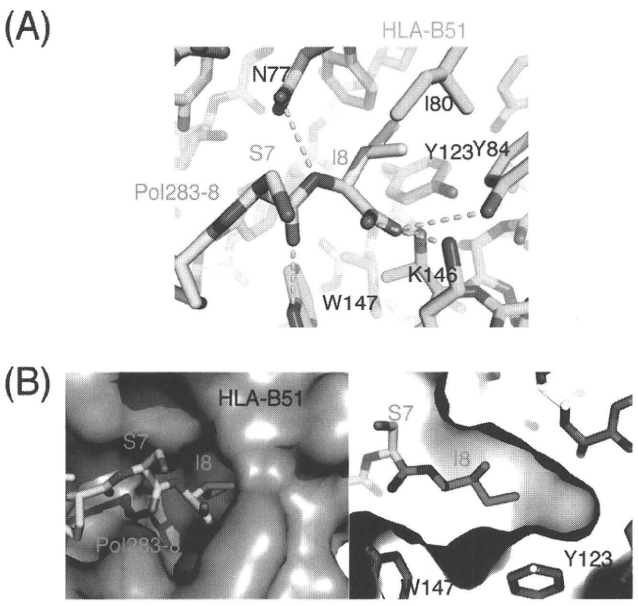

FIG. 6. Binding model of HLA-B^*5101 mutant peptides. (A) Polar interactions around the PC residue in the HLA-B51–Pol283-8 complex. The Pol283-8 peptide and the HLA-B51 heavy chain are shown as yellow and cyan stick models, respectively (N and O atoms are shown as blue and red, respectively). The dotted lines indicate hydrogen bonds or salt bridges. (B) (Left) Surface representation (gray) of the HLA-B51 heavy chain with the stick model of the Pol283-8 peptide (with the same coloring as in panel A). 8I (PC) penetrates into the small pocket. (Right) The sliced image of the small PC pocket (right) explains why bulky and long amino acids are not preferential.

difficulty in controlling the replication of these mutant viruses in HLA-B^*5101^+ individuals initially infected with the mutant virus. We showed previously that recently infected HLA-B^*5101^+ donors have no advantage in the control of HIV-1 (20). Thus, the association between HLA-B^*5101 and slow progression to AIDS may disappear in newly HIV-1 infected Japanese donors.

HLA-B^*57-mediated immune pressure early selects an escape mutant of the TW10 epitope, which has a low viral fitness (29, 32). Escape mutations (K, G, Q, and T at position 242) of the KK10 epitope selected by HLA-B^*27-mediated immune pressure impair viral replication, but the compensatory S173A mutation restores viral replication (40, 41). Pol283-8 escape mutations (T, L, and R) are different from those escape mutations, because these Pol283-8 mutations do not influence viral fitness (43). HLA-B^*5701 is highly associated with LTNPs, but the mechanism of suppression of HIV-1 replication by epitope-specific CTLs still remains unknown (35, 36). On the other hand, several reports indicate that epitope-specific CTLs in HLA-B^*57^+ LTNPs have the ability to cross-recognize variant epitopes (4, 13, 46), suggesting the control of escape mutants by these CTLs. In the present study, we demonstrated the selection of escape mutations by HLA-B^*5101-mediated immune pressure and showed that 2 kinds of mutations, escape mutations for slow progressors and a mutation reducing viral fitness and weakly affecting T-cell recognition for LTNPs, were selected in slow-progressing and LTNP hemophiliacs.

In the present study, we showed that HLA-B^*5101^+ hemo-

philiacs exhibited significantly slow progression during the years 1998 to 2007. Furthermore, we demonstrated that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs was associated with a Pol283-8-specific CD8$^+$ T-cell response. This is the first study finding that a Pol-specific CTL response is more effective in the control of HIV-1 than a Gag-specific CTL response. Our findings provide a novel mechanism for understanding the long-term control of HIV-1 in LTNPs and slow progressors.

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Introduction of TaqMan HIV-1 Assay Increased Unnecessary Drug Resistance Testing

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Dear Editor:

In early 2008, the Roche COBAS TaqMan HIV-1 assay (the TaqMan assay; Roche Diagnostics, Pleasanton, CA) replaced the Roche COBAS Amplicor HIV-1 Monitor version 1.5 (the Amplicor Monitor) for measuring HIV-1 viral load. Although the Amplicor Monitor and the TaqMan assay perform comparably over their respective overall dynamic ranges, there is an increasing concern about poor agreement in measuring low HIV-1 viral load. After the introduction of the TaqMan assay, detectable plasma HIV-1 RNA levels have been reported in a substantial number of infected individuals whose HIV-1 was previously suppressed below the detection limit of the Amplicor Monitor.[1,2] Because reemergence of previously undetectable HIV-1 load while on antiretroviral therapy is a sign of possible virologic failure, the need for therapeutic regimen modification seems necessary and drug resistance testing is reasonable.[3] Therefore, replacement of the Amplicor Monitor by the TaqMan assay might have resulted in unnecessary drug resistance testing. To determine the change in frequency of drug resistance testing in our center, we counted the number of such tests conducted before and after the introduction of the TaqMan assay.

Approximately 1400 HIV-1–infected patients regularly visit our outpatient clinic and approximately two thirds of them are on antiretroviral treatment. The TaqMan assay has been used in our clinic since early March 2008 instead of the Amplicor Monitor.[2] In our clinic, a regular genotypic drug resistance test is normally ordered, unless the HIV-1 load is less than 1000 copies per milliliter, in which case a sensitive genotypic resistance test (preceded by virion concentration with ultracentrifugation) is requested. The frequency of regular genotypic test was 15–52 tests per month between April 2007 and March 2009, and this frequency was similar before and after the introduction of the TaqMan assay (Fig. 1). In comparison, the sensitive genotypic test was requested 0–9 tests per month between April 2007 and April 2008. However, this frequency increased sharply to 37–86 tests per month from May through August 2008. Most of the patients with consistently undetectable HIV-1 load regularly visit the clinic every 2–3 months. It is most likely that their HIV-1 loads were measured by the TaqMan assay for the first time during their visits between March and May 2008. The next visit was probably between May and August 2008, after the results of the first TaqMan assay became available and HIV-1 load was unexpectedly detected in a substantial proportion of the patients. The detected HIV-1 levels should have been less than 1000 copies per milliliter in most cases to warrant requesting the sensitive genotypic resistance test, resulting in the unusually high number of such requests during that period, though the frequency of regular resistance test did not change. After the physicians were made aware that the detection of low-level HIV-1 was common by the TaqMan assay during successful treatment, the frequency of requests for the

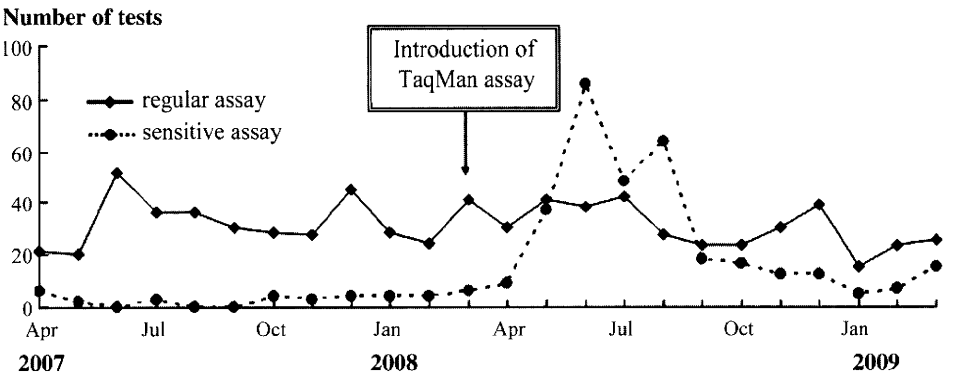

FIG. 1. The frequency of HIV-1 drug resistance tests. The monthly numbers of genotypic resistance tests conducted at the AIDS Clinical Center, International Medical Center of Japan, are shown. Usually, when the HIV-1 load is less than 1000 copies per milliliter, the sensitive resistance test is ordered. Otherwise, a regular resistance test is ordered.

AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan.

sensitive resistance test decreased since September 2008 (5–18 tests per month), although it was still higher compared to that before April 2008. Thus, the introduction of the TaqMan assay resulted in an increase in unnecessary resistance testing. In fact, no emergence of resistance mutations was identified by the sensitive resistance test between March and August 2008. Therefore, clinicians should be notified about which assay is being used to measure their patients' HIV-1 viral load, and they should be aware of the properties of the assays in order to lessen the number of unnecessary resistance tests.

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Original article

Impact of CRF01_AE-specific polymorphic mutations G335D and A371V in the connection subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on susceptibility to nucleoside RT inhibitors

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Abstract

Certain mutations in the connection subdomain and RNase H domain of reverse transcriptase (RT) of subtype B HIV-1 contribute to resistance to nucleoside reverse transcriptase inhibitors (NRTIs). However, the impact of non-B subtype polymorphisms in this region on drug resistance remains unclear. In this study, we determined the frequencies of drug resistance mutations of the entire RT in patients with treatment failure from a cohort of Circulating recombinant form (CRF) 01_AE HIV-1-infected patients in Hanoi, Viet Nam. Subsequently, we assessed the impact of CRF01_AE polymorphisms G335D and A371V with or without thymidine analogue mutations (TAMs) on susceptibility to NRTI with recombinant viruses. In 49 patients with treatment failure, resistance mutations to NRTIs in the N-terminal half of RT were observed in 89.8%. In the C-terminal half, G335D (100%), N348I (36.8%), A371V (100%), A376S (5.3%) and A400T (97.4%) were detected, although G335D, A371V and A400T were considered polymorphisms of CRF01_AE. Drug susceptibility showed G335D, A371V, or both did not confer resistance by themselves but conferred significant resistance to NRTIs with TAMs, especially in mutants containing G335D, A371V and TAM type 2. Our results suggest the important role of CRF01_AE polymorphisms in the C-terminal half of RT in drug resistance.

Keywords: Drug resistance; Reverse transcriptase; G335D; A371V; CRF01_AE

1. Introduction

In Viet Nam, where the epidemic of human immune deficiency virus type 1 (HIV-1) has been in a rapid growth phase with an estimated number of HIV-1-infected individuals rising from 122×10^3 in 2000 to 283×10^3 in 2006, the intensive introduction of antiretroviral therapy (ART) has been implemented with two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) [1,2] and ART coverage of HIV-1-infected individuals has increased from 1% in 2003 to 28.4% in 2007 [3–5]. At the same time, concern regarding drug resistance has emerged [6].

HIV-1 reverse transcriptase (RT) is a heterodimer of two subunits: a 66-kDa subunit (p66) and a 51-kDa subunit and the p66 contains the N-terminal polymerase (codons 1–321), the connection subdomain (codons 322–440) and RNase H (codons 441–560). Although the majority of commercially available genotypic and phenotypic assays have not targeted

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-terminal half of RT: the connection subdomain and
e H domains, certain mutations in this region have been
ly found to be associated with resistance to NRTIs and
Is [7–20]. Despite the accumulation of data on the
ence and mechanisms of mutations in the C-terminal
f RT, most data are from subtype B viruses and little
nation is available on those of non-B subtype. Since
acid sequence diversity in the *pol* gene is 10–15%
g subtypes [21–23] and the subtype have an impact on
esistance mutations [19–31], there is a need to deter-
whether inter-subtype diversity influences the spectrum
g resistance mutations in the C-terminal half of RT as

culating recombinant form (CRF) 01_AE is the most
minant subtype in Viet Nam [32–34] and accounting for
of all HIV-1 infections in Southeast Asia [28–34].
tly, Delviks-Frankenberry demonstrated that the substi-
A400T, a common polymorphism in RNase H of
1_AE, is responsible for the high AZT resistance [16],
gh A400T usually emerged after AZT exposure in
e B [19]. As well as A400T, we found a high frequency
35D and A371V in treatment-naïve CRF01_AE patients
Although G335D and A371V are assumed as common
orphisms in CRF01_AE in the Stanford HIV Drug
ance Database [http://hivdb.stanford.edu/index.html,
ed as late as July 20th 2010], they are thought to be
ated with AZT resistance in subtype B [7,11]. However,
le of substitutions G335D and A371V in drug resistance
TIs has not been well characterized.
the present study, we first investigated drug resistance
ions of CRF01_AE HIV-1 including the connection
main and RNase H domain of RT from HIV-1-infected
ts failing ART. In addition, since we again found high
ncies of the double mutation of G335D and A371V in
opulation, we examined phenotypic resistance levels of
mutations by using mutant recombinant viruses con-
g G335D, A371V or both with or without TAMs, to
nine the impacts of these mutations on drug resistance.

terials and methods

tudy population

V-1-infected patients who had taken antiretroviral
y for more than 6 months at the National Hospital for
al Diseases (NHTD) in Hanoi between October 1, 2007
ne 30, 2008, were enrolled in this study. Each partici-
rovided a written informed consent. Plasma viral load
was measured by the Cobas AmpliPrep-Cobas TaqMan
n (Roche Diagnostics, Tokyo, Japan) and plasma
es were stored at −80 °C for genotypic resistance
g. When pVL was >1000 copies/ml, the patient was
d as treatment failure and the frozen plasma was shipped
e National Center for Global Health and Medicine
M) in Tokyo for genotypic resistance testing.
e study protocol was approved by the institutional ethical
v boards of NTHD and NCGM (IMCJ-H18-360) and by the ethics committee of the Vietnamese Ministry of Health (#1468,1469/QD-BYT dated April 19, 2007).

2.2. Reagents and cells

AZT, stavudine (d4T) and didanosine (ddI) were purchased from Sigma (St. Louis, MO). Lamivudine (3 TC) and tenofovir (TDF) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Abacavir (ABC) was generously provided by GlaxoSmithKline (Philadelphia, PA). Cos-7 and MAGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-Gal cells) were cultured and used as described previously [35].

2.3. Genotypic resistance and subtype analysis

Drug resistance genotyping was carried out by in-house protocols in NCGM. In brief, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription-polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify nearly the entire RT region (codons 1–560) and protease region. The primer sets for amplification of the N-terminal half of RT (codons 1–318) were T1-AE [5′-AGGGGGAATTGGAGGTTT; nucleotides (nt) 2393–2410] and T4-AE (5′-TTCTGTTAGTGCTTTGGTT; nt 3422–3404) for the first PCR, and T12-AE (5′-CCAGTAAAATTAAAGCCAG; nt 2574–2592) and T15-AE (5′-TCCCACTAACTTCTGTATGTC; nt 3335–3315) for the second PCR. The primer sets for amplification of the C-terminal half of RT (codons 319–560) were 3120F-AE (5′-TCTGATTTAGAAATAGGGCAG; nt 3120–3140) and 4428R-AE (5′-GTGTGC AATCTAATTGCCATAT; nt 4428–4407) for the first PCR, and 3240F-AE (5′-GGATATGAACTCCATCCTGA; nt 3240–3259) and 4316R-AE (5′-GTGGCAAATTAAAATCACTAGCC; nt 4316–4295) for the second PCR. Primer sets for amplification of protease were PR01-AE (5′-CCAACAGCCCCACCAGC; nt 2152–2168) and PR02AE (5′-ATTTTCAGGCCCAATT TTTGA; nt 2711–2691) for the first PCR, and PR03-AE (5′-AGCAGGAGCAGAAAGACAAGG; nt 2213 to) and PR04-AE (5′-CTGGCTTTAATKTTACTGGTA; nt 2592–2572) for the second PCR. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 8.0 (Software Development, Tokyo).

Resistance-associated mutations in the N-terminal half of RT were identified according to the International AIDS Society Resistance-USA Panel revised in December 2009 [36] and subtypes of HIV-1 in RT gene were determined by software "Genotyping/NCBI" using BLAST algorithm [http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi].
Resistant mutations in the connection subdomain and the RNase H domain of RT in the previous reports were determined if greater than three-fold increase of EC_{50} compared to

of NL4-3 was noted in the reports. Since all sequences of study participants belonged to CRF01_AE subtype, data requencies of each mutation in the C-terminal half of RT CRF01_AE and subtype B in treatment-naïve patients was ained from the Stanford HIV Drug Resistance Database ://hicdb.stanford.edu/index.html, accessed as late as July , 2010] for reference. Nucleotide sequences of the rminal half of CRF01_AE RT from 38 patients have been osited in the DDBJ database (accession numbers 545813–AB545850).

Construction of recombinant HIV-1 harboring 5D and/or A371V with or without TAMs

o examine the influence of G335D and A371V on drug eptibility to NRTIs, we constructed mutant HIV-1 mbinants that included G335D, A371V or both with or out TAMs. TAM-1 virus was constructed as combina- of M41L, L210W and T215Y and TAM-2 as combina- of D67N, K70R and T215F. Mutant recombinant mid clones of the virus were generated by oligonucleo- site-directed mutagenesis as described previously [10], g pBS-RT_{WT}, which contains the entire RT coding ences (amino acid position 14–560) and three silent riction sites (XmaI, NheI and XbaI from the 5′ to 3′ end RT at codons 15, 267, and 560). After site-directed agenesis, the mutated RT was ligated into pNL4-3, ch contains the entire genome of HIV-1 and the same t restriction sites as pBS-RT_{WT}. The infectious virus generated by transfection of each molecular clone into -7 cells, harvested and stored at −80 °C until use. ctivity was measured as blue cell-forming units (BFU) MAGIC-5 cells. All mutations in recombinant viruses e confirmed by full-length sequencing of the entire RT ing region.

Drug susceptibility assay

usceptibility to NRTIs was determined by using MAGIC- ells as described previously [35] in more than three eriments. MAGIC-5 cells were infected with diluted virus k (100 BFU) in the presence of increasing concentrations TIs, cultured for 48 h, fixed and stained with X-Gal (5- no-4chloro-3-indolyl-βD-galactopyranoside). The stained s were counted under a light microscope. Drug concen- ons that reduced the cell count to 50% of that of the drug- control (EC_{50}) were determined by referring to the e–response curve.

Statistical analysis

Data are expressed as mean ± SD. The Student's t-test was l to compare two groups of continuous variables and value less than 0.05 was considered statistically signifi- . Statistical analyses were performed using SPSSII soft- e package for Windows, version 11.0J (SPSS Japan Inc, yo, Japan).

3. Results

3.1. Characteristics of patients failing antiretroviral therapy

A total of 416 individuals on ART were consecutively enrolled in the present study and their pVLs were assayed between October 1, 2007 and June 30, 2008 at the NTHD in Hanoi. Among them, 49 individuals were confirmed as treatment failure by the definition described above and assigned for genotypic resistance analysis. The characteristics of the 49 individuals are listed in Table 1. All patients had received AZT or d4T plus 3TC combined with NVP, EFV or lopinavir/ritonavir (LPV/r) at the time of enrollment. The most frequently used combination was AZT, 3TC, and NVP, followed by d4T, 3TC and NVP. Protease inhibitors (PIs) were used by 17 (34.7%) patients, while the Vietnamese national ART guideline recommends d4T, 3TC plus 1 NNRTI for the first line regimen [2]. This was probably due to the inclusion of patients who had started ART when the guideline had not been issued yet. The median duration of ART exposure was 2.98 years (IQR 2.17–4.00).

3.2. Genotypic resistance patterns including C-terminal domain of RT

We successfully amplified the N-terminal half of RT and protease of all the 49 patients and C-terminal region of RT of 38 patients. The proportion of patients with at least one NRTI resistance mutation in the N-terminal half of RT was 89.8%.

Table 1
Characteristics of patients failing antiretroviral therapy.

		$n = 49$	(%)
Sex, *n* (%)			
males		33	(67.3)
females		16	(32.7)
Median Age, years (range)		31	(21–50)
Risk of HIV-1 infection (multiple choice), *n* (%)			
sexual contact		46	(93.9)
intravascular drug use		20	(40.8)
CD4 count, median cells/mm^3 (IQR)		145	(84–195)
Plasma viral load, median log copies/ml (IQR),		4.23	(3.59–4.94)
Duration of prior ART, median years (IQR)		2.98	(2.17–4.00)
Experienced ART, *n* (%)			
NRTI	AZT	39	(79.6)
	d4T	24	(49.0)
	3TC	49	(100)
	ddI	7	(14.3)
	ABC	2	(4.1)
	TDF	2	(4.1)
NNRTI	NVP	43	(87.8)
	EFV	15	(30.6)
PI	IDV	12	(24.5)
	SQV	6	(12.2)
	LPVr	3	(6.1)

IQR: interquartile range. ART: antiretroviral therapy. NRTI: nucleoside reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor.