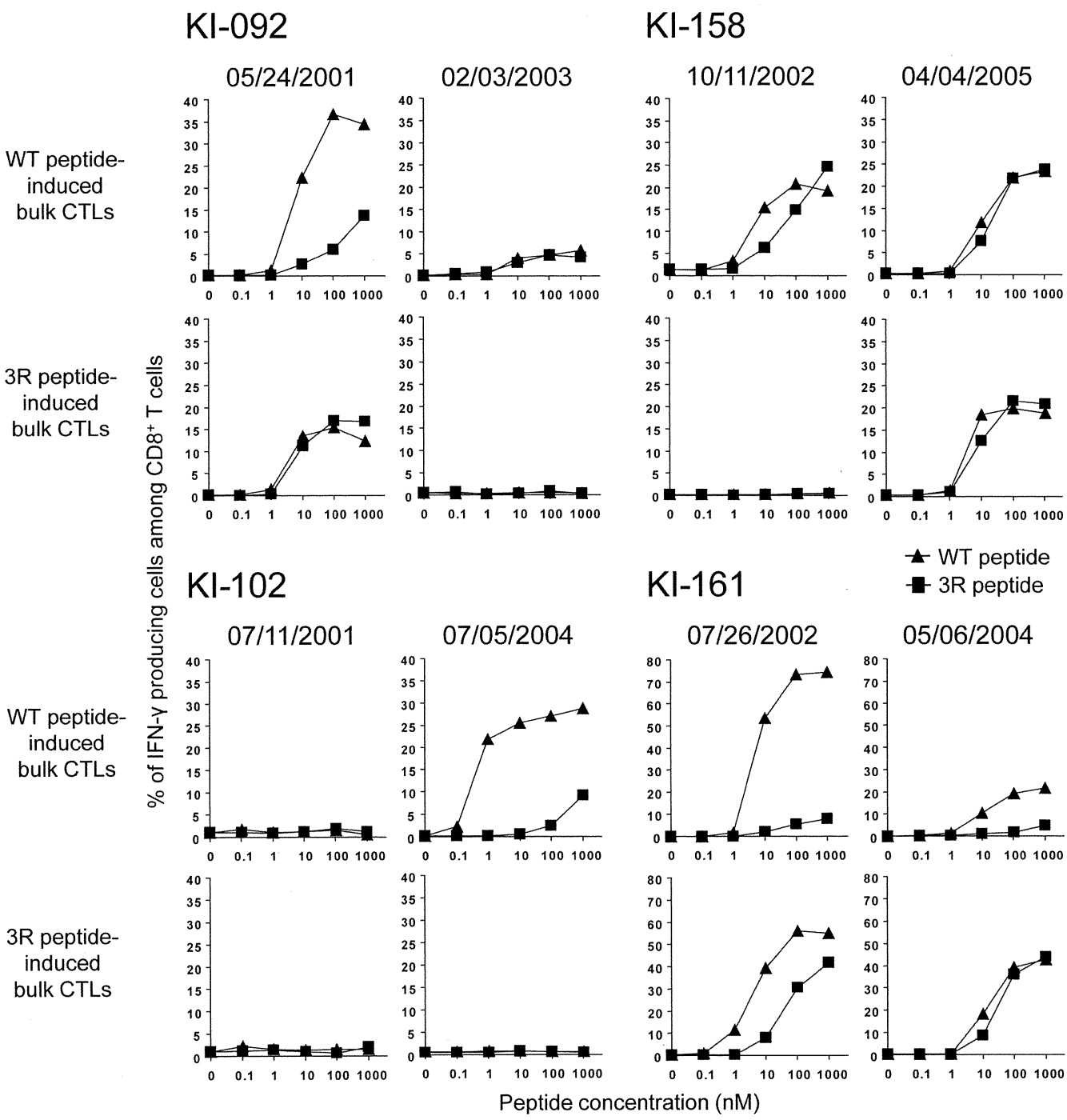

FIG 1 Gag28-specific CD8$^+$ T cells from individuals infected with WT virus at early and chronic phases. Gag28-specific CD8$^+$ T cells were induced by stimulating PBMCs from early and chronic phases in 4 WT-virus-infected HLA-A*24:02$^+$ individuals with WT or 3R peptide. The responses of these bulk-cultured cells to C1R-A2402 cells prepulsed with WT or 3R peptide at concentrations of 0.1 to 1,000 nM were analyzed by using the ICC assay.

graphs under early phase). Then, we analyzed the abilities of these CTL clones to suppress HIV-1 replication. Both WT-specific and cross-reactive CTL clones effectively suppressed the replication of the WT virus, whereas WT-specific and cross-reactive CTL clones exhibited no and weak ability, respectively, to suppress that of the 3R virus (Fig. 3D). These results indicate that WT-specific and cross-reactive CTLs could suppress the replication of the WT virus but that the former CTLs could not suppress the 3R virus *in vivo*. The latter CTLs may weakly suppress 3R virus *in vivo*. Interestingly, the WT-dominant CTL clones exhibited much weaker ability to suppress the replication of WT virus than did the WT-specific and cross-reactive CTLs (Fig. 3D), although no difference in killing activity against WT-virus-infected cells was found among these 3 CTL clones. Overall, KI-161 had a multiple Gag28-

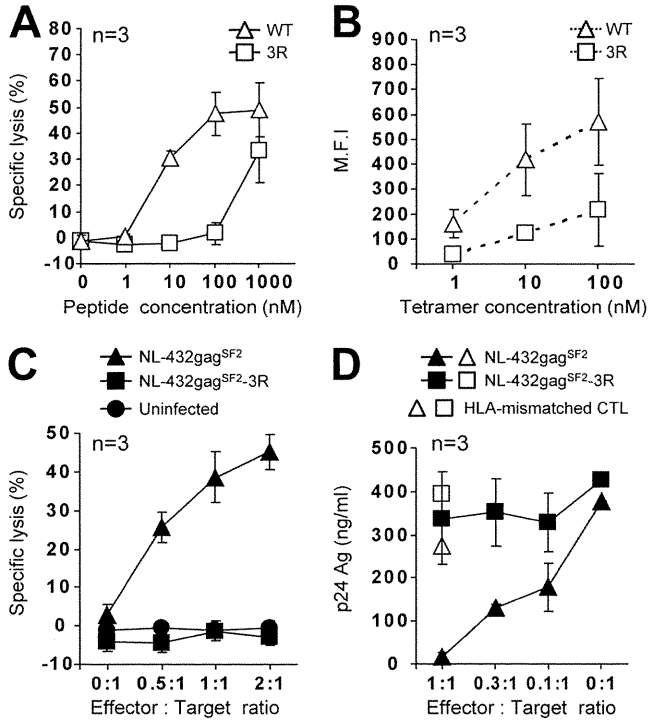

FIG 2 Antiviral activity of Gag28-specific CTL clones generated from early-phase PBMCs from patient KI-092, infected with WT virus. Gag28-specific CTL clones were generated from early-phase PBMCs from KI-092 by stimulating them with WT peptide. The activities of 3 CTL clones (n = 3) were analyzed. (A) Cytotoxic activity toward 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity to WT and 3R tetramers at concentrations of 1 to 100 nM. The MFI values of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with NL-432gag^{SF2} (WT virus) or NL-432gag^{SF2}-3R (3R virus). WT-virus-infected (49.1% of total cells were p24 Ag$^+$) and 3R virus-infected (48.6% of total cells were p24 Ag$^+$) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R viruses. The ability was tested at different E:T ratios. The error bars indicate standard deviations.

specific CTL repertoire at an early phase of HIV-1 infection, but only 2 types of Gag28-specific CTLs, which were the majority among the Gag28-specific CTLs, contributed to the suppression of WT virus replication.

Cross-reactive CD8$^+$ T cells and 3R-specific CD8$^+$ T cells in individuals who were infected with 3R virus. Next, we analyzed the elicitation of Gag28-specific CD8$^+$ T cells in 5 individuals infected with the 3R virus. Gag28-specific CD8$^+$ T cells were detected at both early and chronic phases in 3 individuals, whereas they were found at only the chronic phase in the other 2 (Table 3). Cross-reactive CD8$^+$ T cells were induced by stimulating KI-091 PBMCs from both early and chronic phases, not only with 3R peptide, but also with WT peptide. To characterize Gag28-specific CD8$^+$ T cells in KI-091, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-091 by stimulating them with 3R peptide. We investigated the recognition of 3 CTL clones for WT and 3R peptides. These CTL clones evenly recognized both peptides (Fig. 4A) and revealed the same binding affinity for the 2 tetramers (Fig. 4B), indicating that they were cross-reactive CTLs. They moderately killed target cells infected with either WT or 3R virus (Fig. 4C) but did not suppress the replication of the WT and 3R viruses (Fig. 4D). Thus, Gag28-specific CD8$^+$ T cells elicited in KI-091 had no ability to suppress the replication of WT and 3R viruses. Further analysis of 13 other clones revealed similar characteristics (data not shown), supporting the data indicating that cross-reactive CTLs were predominantly elicited in KI-091.

In the chronic phase, KI-091 had cross-reactive CD8$^+$ T cells, whereas 3R-specific CD8$^+$ T cells were found in 4 other individuals (Table 3). To characterize these 3R-specific CD8$^+$ T cells, we generated 3R-specific CTL clones from KI-163 PBMCs at the chronic phase by stimulating them with 3R peptide. All 3 clones recognized the 3R peptide much more effectively than the WT peptide (Fig. 4A). These CTL clones bound to 3R tetramer, but not to WT tetramer (Fig. 4B), indicating that these CTL clones carried a 3R-specific TCR. In addition, we analyzed the abilities of these CTL clones to recognize virus-infected cells and found that they effectively killed target cells infected with 3R virus, but not those infected with WT virus (Fig. 4C). However, they failed to suppress the replication of either 3R or WT virus (Fig. 4D). These results indicate that Gag28-specific CD8$^+$ T cells elicited in all individuals infected with 3R virus had no ability to suppress the replication of WT or 3R virus. Thus, Gag28-specific CD8$^+$ T cells seem to have failed to control the 3R virus, although they were elicited in individuals infected with the 3R virus.

High accumulation of the 3R variant in the Japanese population. The results described above strongly suggest that WT-specific and cross-reactive CD8$^+$ T cells selected the 3R mutation in the individuals infected with the WT virus and that 3R-specific and cross-reactive CD8$^+$ T cells failed to control the 3R virus in the individuals infected with it. Therefore, we assume that this 3R mutation has accumulated in the HLA-A*24:02$^+$ individuals. In addition, since HLA-A*24:02 is found in approximately 70% of Japanese, we speculate that the mutation has accumulated to high levels in the Japanese population.

A previous study analyzed the frequency of 3R in only 32 HLA-A*24:02$^+$ and 26 HLA-A*24:02$^-$ individuals chronically infected with HIV-1 and showed that the frequency of 3R was significantly higher in HLA-A*24:02$^+$ individuals than in the HLA-A*24:02$^-$ individuals (30). To confirm the association of this mutation with HLA-A*24:02, we analyzed a large number of chronically HIV-1-infected nonhemophiliac individuals (220 HLA-A*24:02$^+$ and 154 HLA-A*24:02$^-$ individuals) recruited from April 2008 to March 2011 (2008 to 2011 cohort). The results confirmed that the frequency of 3R was significantly higher in HLA-A*24:02$^+$ individuals than in the HLA-A*24:02$^-$ individuals ($P < 0.0005$) (Fig. 5). Since 3R was found in 74.7% of the HLA-A*24:02$^-$ individuals in this cohort, we speculate that the mutation has been accumulating in the Japanese population. Therefore, we analyzed HIV-1-infected nonhemophiliac Japanese individuals who had been recruited from 1996 to 2002 (1996 to 2002 cohort), as well as Japanese hemophiliacs who had been infected around 1983 (hemophiliac cohort), and then compared them to the 2008 to 2011 cohort (Fig. 5). The association of this mutation with HLA-A*24:02 was also found in both the 1996 to 2002 cohort and the hemophiliac cohort ($P < 0.01$ and $P = 7.4 \times 10^{-7}$, respectively). The frequency of this mutation in HLA-A*24:02$^-$ individuals significantly increased from 0% in the hemophiliac cohort to 50.0% in the 1996 to 2002 cohort ($P = 0.0084$) and to 74.7% in the 2008 to 2011 cohort ($P = 2.6 \times 10^{-7}$). These results indicate that the 3R mutation was strongly selected by Gag28-specific CTLs and has

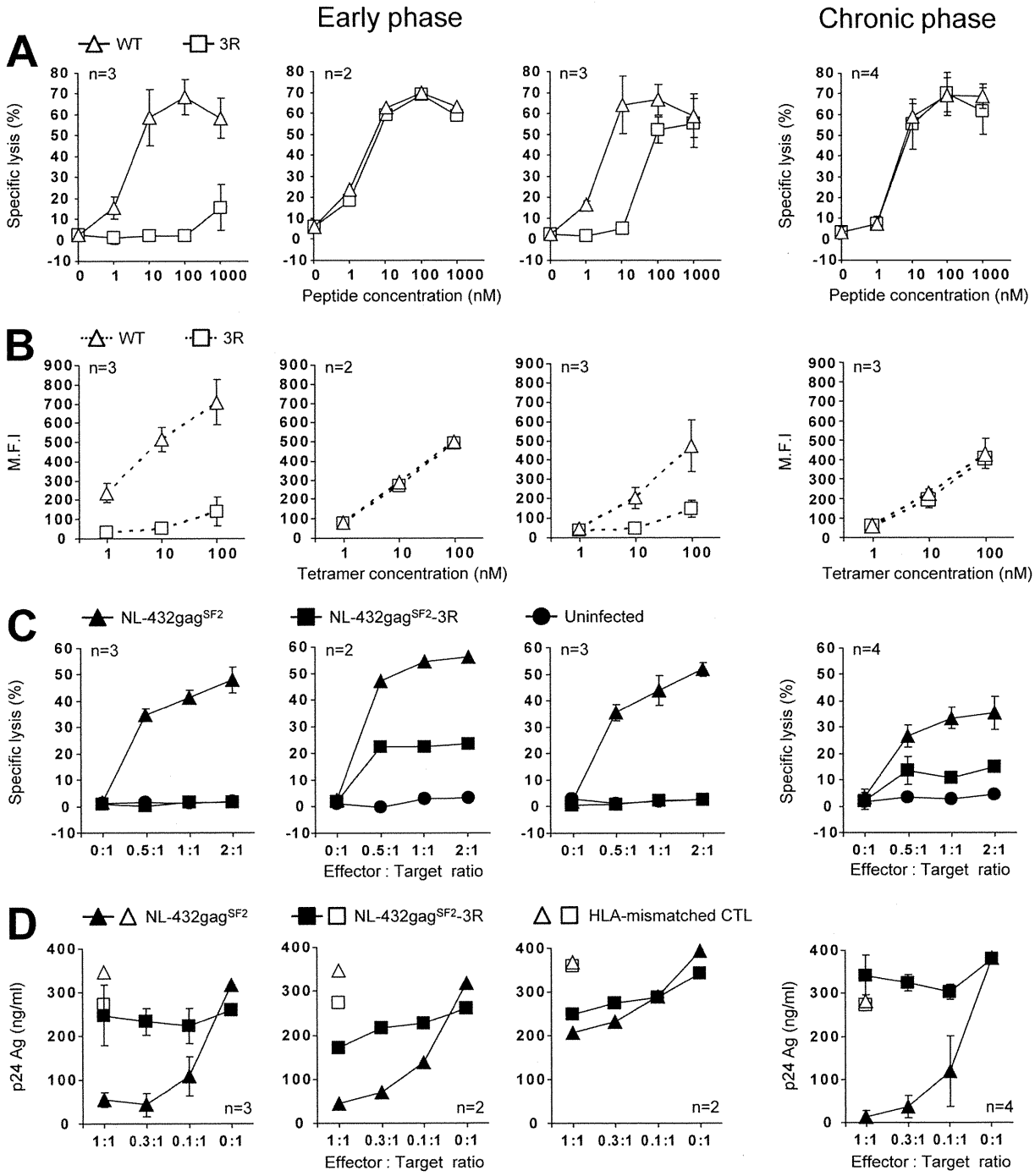

FIG 3 Antiviral activities of Gag28-specific CTL clones generated from PBMCs of patient KI-161, infected with WT virus. Gag28-specific CTL clones were generated from early-phase and chronic-phase PBMCs isolated from KI-161 after stimulating them with the WT and 3R peptides, respectively. Three types of Gag28-36-specific CTL clones, i.e., WT specific (left), cross-reactive (middle), and WT dominant (right), were generated from the early-phase PBMCs. (A) Cytotoxic activity against 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity toward WT and 3R tetramers at concentrations of 1 to 100 nM. The MFIs of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with WT virus or 3R virus. WT-virus-infected (49.0% of total cells were p24 Ag$^+$) and 3R-virus-infected (50.0% of total cells were p24 Ag$^+$) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R virus. The ability was tested at different E:T ratios. n, number of clones tested. The error bars indicate standard deviations.

been accumulating during the past 30 years in the Japanese population.

It is well known that some escape mutations affect replication capacity and that HIV-1 containing such mutations reverts to WT in individuals not carrying HLA class I restriction alleles (23, 32). We previously showed that the 3R mutation does not affect replication capacity when 2 T cell lines are used in an assay measuring it (46). Since a different effect of mutations on replication capacity

TABLE 3 Responses of CD8$^+$ T cells from individuals infected with 3R virus to WT or 3R peptide

Patient ID	Virus sequence [mo/day/yr (type)]		PBMC sampling date (mo/day/yr)	PBMCs cultured with:	% IFN-γ-producing cells specific for each peptide among CD8$^+$ T cells[a]		
	Early phase	Chronic phase			Without	WT	3R
KI-091	12/13/2000 (3R)	8/4/2005 (3R)	12/13/2000	WT	0.2	**74.6**	**71.2**
				3R	0.3	**55.4**	**71.9**
			9/29/2004	WT	0.2	**77.7**	**65.5**
				3R	0.2	**61.1**	**69.3**
KI-134	10/25/2001 (3R)	6/30/2004 (3R)	10/25/2001	WT	0.4	0.6	0.8
				3R	1.0	1.1	**5.7**
			1/21/2004	WT	0.8	1.0	0.7
				3R	0.7	0.6	**2.0**
KI-136	10/29/2001 (3R)	7/10/2003 (3R)	10/29/2001	WT	0.1	0.4	0.2
				3R	0.1	0.2	0.2
			5/15/2003	WT	0.4	0.8	0.4
				3R	0.1	0.2	**24.8**
KI-151	2/15/2002 (3R)	6/16/2005 (3R)	11/21/2001	WT	0.3	0.7	0.8
				3R	0.7	0.6	**10.8**
			7/28/2004	WT	0.4	0.7	1.3
				3R	0.1	0.1	**44.5**
KI-163	8/30/2002 (3R)	9/27/2004 (3R)	8/30/2002	WT	0.2	0.3	0.2
				3R	0.2	0.4	0.2
			8/29/2005	WT	0.3	0.5	0.2
				3R	0.4	0.6	**6.9**

[a] Without, without peptide. Boldface, positive IFN-γ-producing response.

between cell lines and CD4$^+$ T cells from a healthy individual is known (23), we measured the replication capacity of the 3R virus by using CD4$^+$ T cells from a healthy individual. The results confirm that this mutation did not affect the replication capacity (Fig. 6), suggesting that the 3R mutant could not revert in HLA-A*24:02$^-$ individuals.

DISCUSSION

It is known that CTLs recognizing escape mutants are elicited after the escape mutant had been selected by WT epitope-specific CTLs (2, 4, 12, 15, 33, 39) or in new escape mutant virus-infected hosts having the same restricted HLA allele (15). However, since the CTLs recognizing escape mutants have been not well analyzed, the role of these CTLs in the control of HIV-1 infections remains unclear. In the present study, we investigated 2 groups, HLA-A*24:02$^+$ individuals infected with WT virus and those infected with 3R escape mutant virus. We found that both WT-specific and cross-reactive CD8$^+$ T cells were elicited in individuals infected with WT virus. Interestingly, cross-reactive T cells had been elicited before the emergence of the 3R escape mutant virus, though a similar finding was made in previous studies that analyzed other epitope-specific CTLs (18, 25, 26, 34). The present study shows that WT-specific CD8$^+$ T cells were predominantly elicited in an early phase of the infection and that the number of cross-reactive CD8$^+$ T cells increased in the chronic phase. The CTL clones from early and chronic phases in KI-161 showed similar abilities to kill WT virus-infected or 3R virus-infected cells and activities to suppress both viruses, suggesting that cross-reactive CD8$^+$ T cells elicited at the early phase were expanded via antigen presentation by 3R virus-infected cells at the chronic phase.

WT-specific and cross-reactive CTL clones from KI-092 and KI-161 at an early phase of the infection effectively killed WT-virus-infected cells and suppressed the replication of the WT virus, whereas they exhibited no and weak ability, respectively, to suppress that of the 3R virus. Cross-reactive CTL clones had the same ability to suppress the replication of WT virus as did the WT-specific CTL clones. These results strongly suggest that both CTLs selected the 3R virus in these individuals infected with the WT virus. The 3R virus was not selected within at least 1 year after Gag28-specific CTLs had been detected in the individuals infected with the WT virus. This finding indicates that the 3R mutation was more slowly selected by these CTLs than escape mutants selected at an acute phase of the infection (16, 19, 34, 44, 45). On the other hand, a previous study suggested that acute accumulation of mutations in this epitope occurs after an HIV-1 infection (6). However, the data shown in that study concerned mutations contained at position 1 of the epitope. In addition, those data may have included cases in which the individuals had been infected with the 3R mutant virus, because it may be assumed that 3R virus had accumulated in the cohorts analyzed. Cross-reactive CTL clones established from PBMCs at both early and chronic phases of KI-161 killed 3R virus-infected cells, though the killing activity against the 3R virus-infected cells was weaker than that against the WT virus-infected cells. These CTL clones weakly suppressed the replication of the 3R virus (Fig. 3C). This weak ability to suppress it might have delayed the emergence of the 3R mutation in these patients.

WT-specific CTLs were not induced by stimulation of early- or chronic-phase PBMCs from the 5 individuals in which the 3R mutation had been detected at the early phase with WT peptides. This finding supports the possibility that these individuals had been infected with the 3R virus. Only KI-091 had cross-reactive T cells at early and chronic phases of the infection. All CTL clones established from this patient had cross-reactivity, implying that the patient had been infected with WT virus and that 3R had been selected at an early phase. However, WT-specific CTL clones were not established from this patient. In addition, the cross-reactive

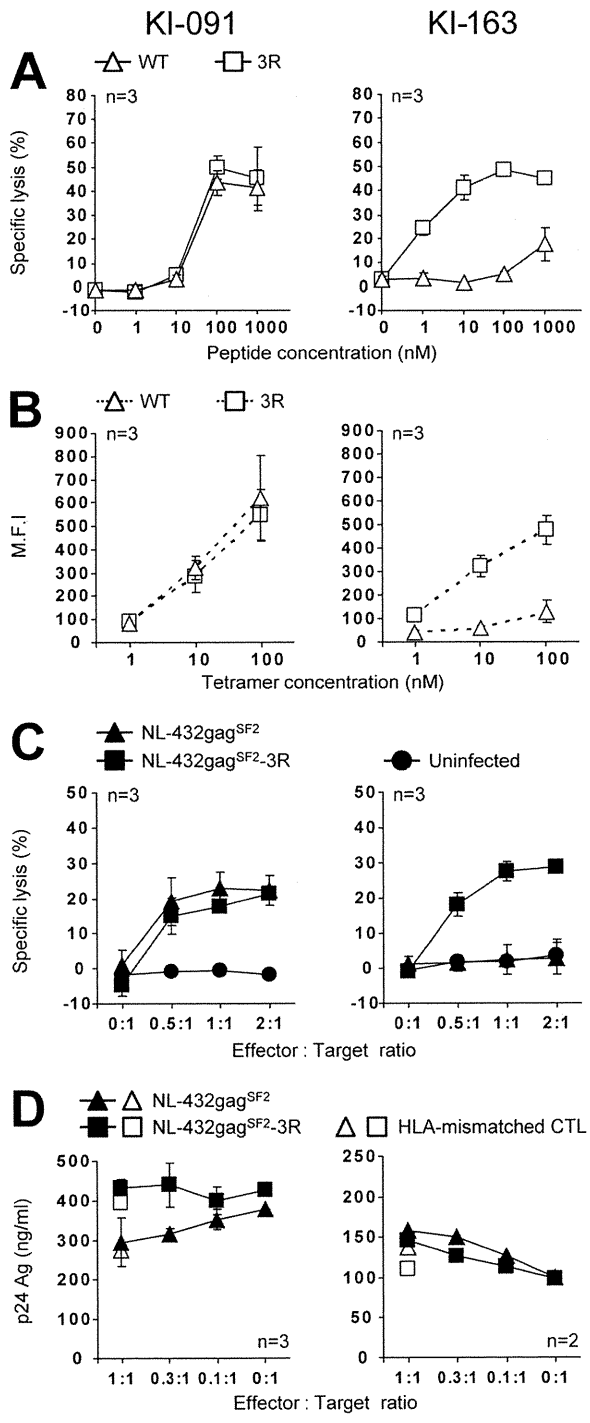

FIG 4 Antiviral activities of cross-reactive and 3R-specific CTL clones generated from patients KI-091 and KI-163 infected with 3R virus. Gag28-specific CTL clones were generated from chronic-phase PBMCs isolated from patients KI-091 and KI-163 after their stimulation with 3R peptide. The following activities of these CTL clones were analyzed. (A) Cytotoxic activity against 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity toward WT and 3R tetramers at concentrations of 1 to 100 nM. The MFIs of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with WT virus or 3R virus. WT-virus-infected and 3R virus-infected cells were used as target cells. The frequency of p24 Ag$^+$ cells among the HIV-1-infected cells was as follows: WT-virus-infected cells, 49.1% and 43.1% for CTL clones from KI-091 and KI-163, respectively, and 3R-virus-infected cells, 48.6% and 45.6% for CTL clones from KI-091 and KI-163, respectively. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R virus. The abilities were tested at different E:T ratios. n, number of clones tested. The error bars indicate standard deviations.

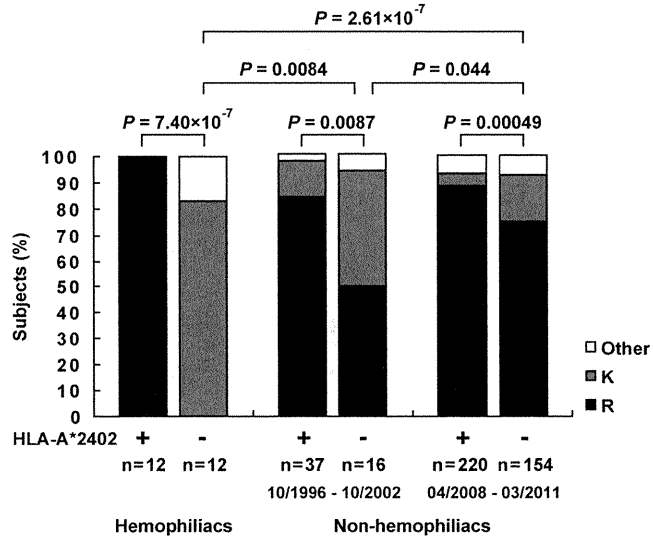

FIG 5 Frequencies of the 3R mutation in a Japanese hemophiliac cohort and nonhemophiliac cohorts recruited from 1996 to 2002 and from 2008 to 2011. The frequencies of mutations at position 3 of the Gag28 epitope in chronically HIV-1-infected HLA-A*24:02$^+$ or HLA-A*24:02$^-$ hemophiliac individuals and nonhemophiliac individuals recruited from 1996 to 2002 or from 2008 to 2011 are shown. The consensus sequence of this epitope in HIV-1 subtype B is KYKLKHIVW. The frequency of the 3R mutation between HLA-A*24:02$^+$ and HLA-A*24:02$^-$ subjects in each cohort or that in HLA-A*24:02$^+$ or HLA-A*24:02$^-$ subjects among the 3 cohorts was statistically analyzed by using Fisher's exact test.

CTL clones established from KI-091 did not have the ability to suppress the replication of the WT virus, although the CTL clones from individuals who had been infected with the WT virus had strong ability to suppress it. These findings suggest that this patient had been infected with the 3R virus rather than with the WT virus. However, it remains unknown why 3R-specific CTLs were elicited in the other 4 individuals but not in this patient. Thus, the abilities of CTLs to respond to WT peptide and to suppress the replication of WT virus together supported the idea that the individuals who had 3R virus in the early phase had been infected with 3R virus, although the possibility that they had been infected with WT virus cannot be completely excluded.

The 3R mutant epitope peptide would have been processed and presented to 3R-specific CTLs in 3R virus-infected cells, since 3R-specific and cross-reactive CTL clones effectively killed 3R virus-infected cells. However, these CTL clones failed to suppress the replication of the 3R virus. 721.221-CD4-A2402 cell lines were used as target cells for the killing assay, whereas CD4$^+$ T cells from healthy individuals were used for the replication suppression assay. The former cells express HLA-A*24:02 to a much higher degree than the latter cells. This difference between the 2 cell lines may account for the discrepancy of the results between the 2 assays. 3R-specific CTL clones failed to suppress the replication of the 3R virus, whereas cross-reactive CTLs from the individuals

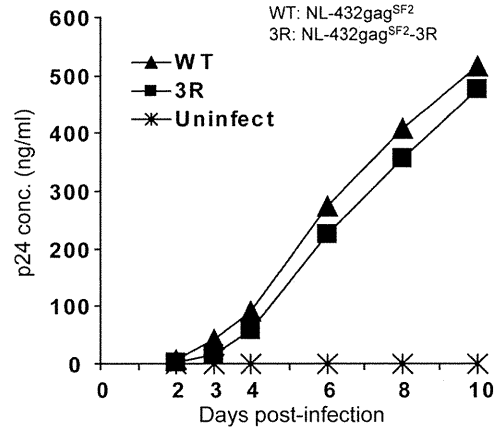

FIG 6 Replication kinetics of WT and 3R viruses in CD4$^+$ T cells. CD4$^+$ T cells (2×10^5) isolated from PBMCs from a healthy donor were infected with WT or 3R virus in triplicate at a blue-cell-forming unit of 500 (in MAGIC-5 cells) in a total volume of 0.2 ml and then incubated at 37°C for 2 h. The infected cells were washed twice with R10 and then cultured in 1 ml of complete medium plus rIL-2 at 37°C. A 0.1-ml volume of the culture supernatants was collected at days 2 to 10 postinfection. The concentration of p24 Ag was measured by using ELISA.

infected with WT virus effectively suppressed the replication of the WT virus but failed to suppress that of the 3R virus. These findings suggest that 3R virus-infected CD4$^+$ T cells could not effectively present the 3R mutant epitope. This finding also suggests that 3R virus-infected CD4$^+$ T cells were not the main source of antigen-presenting cells in 3R virus-infected individuals. A previous study showed that HIV-1-infected macrophages effectively present HIV-1 epitopes more than HIV-1-infected CD4$^+$ T cells (14), implying that 3R virus-infected macrophages are the main antigen-presenting cells and contribute to the elicitation of 3R-specific and cross-reactive CTLs in 3R virus-infected individuals. A further study should clarify the role of macrophages in the elicitation of 3R-specific and cross-reactive CTLs in 3R virus-infected individuals.

Cross-reactive CTLs were found in individuals infected with the WT virus or with the 3R virus. The CTL clones established from individuals infected with the WT virus had a strong ability to kill WT-virus-infected cells and to suppress the replication of the WT virus, whereas those established from an individual infected with the 3R virus showed moderate ability to kill WT-virus-infected cells and no ability to suppress the replication of WT virus. These findings indicate that cross-reactive CTLs from an individual infected with the 3R virus may have had less ability to recognize the WT epitope than those from an individual infected with the WT virus. Indeed, the former CTL clones exhibited lower sensitivity to reaction with WT peptide-pulsed cells than the latter CTLs, indicating that cross-reactive CTLs elicited in individuals infected with the WT virus had higher-affinity TCRs for WT peptide than those in an individual infected with the 3R virus. In addition, the latter CTL clones weakly killed 3R virus-infected cells, whereas the former clones showed the same killing activity against 3R virus-infected cells as against WT-virus-infected cells. Thus, cross-reactive CTLs in individuals infected with 3R virus have different characteristics than those in individuals infected with the WT virus. This finding suggests that cross-reactive CTLs elicited in individuals infected with the WT virus had TCRs with higher affinity for WT and 3R peptides than those in individuals infected with the 3R virus.

Japanese hemophiliacs were infected with HIV-1 via blood products from the United States around 1983, and HLA-A*24:02 is a rare allele in North America. Therefore, it may be speculated that HIV-1 in the blood product had not yet accumulated escape mutations. Indeed, the 3R mutation was not found in the 12 HLA-A*24:02$^-$ hemophiliacs tested, though other amino acid variants at position 3 were detected in 2 of these hemophiliacs. This mutation was found in 50.0% of HLA-A*24:02$^-$ individuals in the 1996 to 2002 cohort and in 74.7% of those in the 2008 to 2011 cohort, indicating that the mutation had accumulated in the Japanese population. The frequency of this mutation in HLA-A*24:02$^-$ individuals thus increased about 1.5-fold during the approximately 10-year period between these 2 nonhemophiliac cohorts. Thus, the mutation greatly accumulated over the last 10 years. Since HLA-A*24:02 is found in approximately 70% of Japanese, the high prevalence of the allele is the cause of the high accumulation of the 3R mutation in the Japanese population. In addition, this high accumulation resulted not only from a strong selection of the 3R mutation by WT-specific and cross-reactive CTLs elicited in the donors infected with WT virus, but also from a lack of reversion of the mutation in the HLA-A*24:02$^-$ individuals.

Our previous study concerning HLA-A*24:02-restricted Nef138-specific CTLs demonstrated that only WT epitope-dominant CTLs, which suppress the replication of WT virus but fail to suppress that of mutant virus, are elicited at an early phase in HLA-A*24:02$^+$ individuals infected with the WT virus and that mutant-epitope-dominant CTLs but not cross-reactive CTLs are elicited after the emergence of the mutant virus in them (15). In addition, only mutant-epitope-dominant CTLs are elicited in those individuals infected with the mutant virus. The mutant-epitope-dominant CTLs suppress the replication of WT virus but weakly suppress that of mutant virus (15). Thus, Nef138-specific CTLs elicited in individuals infected with WT or mutant viruses had different characteristics in terms of the recognition of WT and mutant epitopes than the Gag28-specific CTLs analyzed in the present study. The difference between Nef138-specific and Gag28-specific CTLs might be explained by a different CTL repertoire elicited at an early phase. These 2 studies suggest the elicitation of various HIV-1-specific CTLs in regard to recognition of escape mutations.

In the present study, we demonstrated that WT-specific and cross-reactive CTLs were elicited at an early phase in individuals infected with the WT virus and that cross-reactive CTLs were dominant in Gag28-specific CTLs after the emergence of the 3R virus. On the other hand, 3R-specific and cross-reactive CTLs were elicited in individuals infected with the 3R virus, though the former CTLs were predominantly elicited in these individuals. The CTLs elicited in the individuals infected with the WT virus, which had a strong ability to suppress the replication of WT virus, played a central role in the accumulation of the 3R mutation. In contrast, the CTLs elicited in those infected with 3R virus, which failed to suppress the replication of WT and 3R viruses, did not contribute to the control of the 3R virus infection. In addition, the high prevalence of HLA-A*24:02 and lack of effect of the 3R mutation on viral fitness may have strongly contributed to the high accumulation of the mutation in HIV-1-infeceted Japanese individuals.

ACKNOWLEDGMENTS

This research was supported by the Global COE program Global Education and Research Center Aiming at the Control of AIDS, launched as a project commissioned by the Ministry of Education, Science, Sports, and

Culture, Japan; by a grant-in-aid for scientific research from the Ministry of Health (no. 18390141), Japan; and by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture (no. 20390134), Japan.

The authors have no conflicting financial interests.

We thank Sachiko Sakai for her secretarial assistance.

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Impact of human leukocyte antigen-B*51-restricted cytotoxic T-lymphocyte pressure on mutation patterns of nonnucleoside reverse transcriptase inhibitor resistance

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Objective: The objective of this study is to determine the impact of human leukocyte antigen (HLA)-B*51-restricted cytotoxic T-lymphocyte (CTL) pressure on the development of nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance.

Design: The prevalence of HIV-1 harboring an escape mutation, I135X, in a major epitope of HLA-B*51-restricted CTL located in reverse transcriptase is increasing worldwide. We analyzed the effects of escape mutations on the emerging mutation patterns of NNRTI resistance.

Methods: Monoclonal HIV-1 sequences harboring each of the escape mutations, including I135L (HIV-1_{I135L}), I135V (HIV-1_{I135V}), I135T (HIV-1_{I135T}), and I135R (HIV-1_{I135R}) in reverse transcriptase, and a wild-type monoclonal HIV-1 (HIV-1_{WT}) were cultured in the presence of increasing concentrations of efavirenz. Induced mutations during culture passages of the culture were analyzed.

Results: E138K emerged during the cultural passages of HIV-1_{I135V}, HIV-1_{I135T}, and HIV-1_{I135R}, but not during the passages of HIV-1_{WT}. The combination of I135T, the most frequent escape mutation, and E138K (HIV-$1_{I135T/E138K}$) conferred significant resistance to efavirenz, nevirapine, and etravirine. The HIV-$1_{I135L/E138K}$ and HIV-$1_{I135R/E138K}$ were significantly resistant to nevirapine and etravirine, respectively, though each solo of escape mutations and E138K did not confer significant resistance to NNRTI. Computational analysis indicated that I135T and E138K cooperatively extend the gap between the binding site of reverse transcriptase and NNRTI.

Conclusion: HLA-B*51-restricted CTL can induce novel mutation patterns of NNRTI resistance by selecting escape mutations. The spread of CTL escape variants may alter the mutation patterns of drug resistance.

AIDS 2010, **24**:F15–F22

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Received: 9 November 2009; revised: 28 December 2009; accepted: 19 January 2010.

DOI:10.1097/QAD.0b013e328337b010

ISSN 0269-9370 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins

Keywords: antiretroviral therapy, cellular immunity, cytotoxic T-lymphocyte escape variant, E138K, major epitope

Introduction

Cytotoxic T lymphocytes (CTLs) are one of the antiretroviral host factors that can modify the clinical course of HIV-1 infection [1]. However, HIV-1 evades these cells by acquiring escape mutations in recognized epitopes, and some of the CTL-escape variants remain stable without reversion even in the absence of such selective pressure [2]. TAFTIPSI (reverse transcriptase 128–135) is a major epitope recognized by human leukocyte antigen (HLA)-B*51-restricted CTL [3], and we recently reported that its escape mutation, I135X, is detected in the majority of HLA-B*51-positive infected individuals and also in a significant proportion of HLA-B*51-negative individuals, and that I135X can exist persistently even in HLA-B*51-negative individuals probably because it does not cause a significant fitness cost [4]. Consequently, I135X can spread as a polymorphic mutation among infected individuals and has in fact accumulated in the HIV-positive populations, especially among the Japanese, in whom HLA-B*51 is highly prevalent. Previous studies reported that I135X was associated with low-level resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) [5–7] and suggested that I135X may be a determinant of evolutional patterns of NNRTI resistance [8,9], though it has also been reported that there is no correlation between the presence of I135X at baseline and efficacy of NNRTI [10]. To determine whether CTL escape mutations alter the development of drug resistance, we focused on I135X and induced NNRTI resistance from I135X-harboring HIV-1s by cultural passages in the presence of increasing concentrations of efavirenz (EFV).

Materials and methods

HIV-1 sequences and human leukocyte antigen types in treatment-naive patients

We recently reported the frequent prevalence of I135X mutations in Japan [4]. To confirm the same and to determine the frequency of each mutation, we used another cohort that included 575 treatment-naive newly diagnosed HIV/AIDS patients recruited from across Japan between January 2003 and December 2004 [11]. Among them, data of HLA typing were available for 97 patients.

Generation of recombinant HIV-1 sequences

The desired mutations were introduced into the *Xma*I-*Nhe*I region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 reverse transcriptase (strain BH10) [12]. The *Xma*I-*Nhe*I fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. Each molecular clone was transfected into COS-7 cells, and the obtained virions were harvested 48 h after transfection and stored at −80°C until use.

Induction of efavirenz-resistant HIV-1

The infectious HIV-1 clones were propagated in MT-2 cells in the presence of increasing concentrations of EFV [12]. Briefly, MT-2 cells (1×10^5) were exposed to 500 blue cell-forming units (BFUs) in MAGIC-5 cells (CCR5-expressing and CD4-expressing HeLa-LTR-β-D-gal cells) of each monoclonal HIV-1 and cultured in the presence of EFV at an initial concentration of 3 nmol/l. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased by half-log fold. This selection was carried out until the EFV concentration reached 1000 nmol/l. Proviral HIV-1 reverse transcriptase gene in the infected MT-2 cells was amplified and sequenced at several passages.

Drug susceptibility assay

EFV and nevirapine (NVP) were generously provided by Merck Co., Inc. (Rahway, New Jersey, USA) and Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, Connecticut, USA), respectively. Etravirine (ETR) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Recombinant HIV-1 susceptibility to EFV, NVP, and ETR was determined in triplicate using MAGIC-5 cells [12]. The drug susceptibility assay was performed in triplicate and repeated three times. Fold resistance was calculated by comparing viral IC_{50} with that of monoclonal wild-type HIV-1 ($HIV\text{-}1_{WT}$). Drug resistance was considered significant when it was higher than three-fold.

Structural modeling

We constructed structural models of the HIV-1 reverse transcriptase and NNRTI complex by computational analysis. First, we constructed the initial models of wild-type reverse transcriptase with one of the three NNRTIs by homology modeling using Molecular Operating Environment (MOE) 2007.09.02 (http://www.chemcomp.com/). The crystal structures of reverse transcriptase with NNRTI (PDB code: 1IKW [13], 1VRT [14], and 1SV5 [15]) were used for template structures. The ff94 force field and distance-dependent electrostatic energy function were applied in the modeling. Next,

we refined the initial models by energy minimization using sander module of AMBER9 software package through two steps. In the first step, energies for the NNRTI in the complex models were minimized at the gas phase by the conjugated gradient method. In the second step, energies of whole structures were converged up to 0.5 kcal/mol/Å by 50 steps of the steepest descent method and the subsequent conjugated gradient method at implicit water solvent condition. In each minimization, the AMBER ff03 [16,17], the general AMBER force field (gaff) [18], and the generalized Born implicit solvent surface area (GBSA) method (IGB = 2) [19] were applied for potential energy calculations. The charges and atom types of every atom in NNRTI were automatically assigned using the AMBER9 Antechamber module. We also constructed the respective mutant reverse transciptases with the NNRTI by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL version 0.99rc6 (http://www.pymol.org). The structural model of each conformer was refined by a method similar to that used in the wild-type models. Among the refined conformers, we selected those with the lowest energy as each mutant model.

Results

The 135th amino acid in HIV-1 reverse transcriptase and human leukocyte antigen-B*51

We analyzed the relationship between HLA-B*51 and the 135th amino acid of HIV-1 reverse transciptase in 97 infected individuals newly diagnosed in Japan between January 2003 and December 2004 (Table 1). As expected, CTL escape mutations I135X, including I135T, I135L, and I135V, were observed in all but one HLA-B*51-positive patient (94.1%), representing a significantly higher prevalence than in the HLA-B*51-negative patients (Fisher's exact test; $P = 0.01$). However, in the HLA-B*51-negative patients, escape mutations were still observed at a high frequency (62.5%), indicating that I135X variants can transmit from HLA-B*51-positive patients to HLA-B*51negative individuals and can persist even in the absence of HLA-B*51-restricted CTL pressure. Overall, I135X mutations were observed at a high frequency in the treatment-naive patients in Japan, and the most frequent amino acid was I135T (35.1%), which was more frequent than the wild-type I135 (32.0%).

Induction of efavirenz-resistant HIV-1

As described above, I135L, I135V, I135T, and I135R mutations were detected in treatment-naive patients. In order to analyze their effects on the mutation pattern for NNRTI resistance, EFV resistance was induced from monoclonal HIV-1s harboring each of these mutations by culturing them in the presence of increasing concentrations of EFV. These induction experiments were performed independently in triplicate. In one of the three induction experiments on HIV-1_{I135L}, V179D emerged when EFV concentration reached 100 nmol/l, as well as emergence of K103R in the presence of EFV at 1000 nmol/l (Fig. 1a). We previously reported that the combination of K103R and V179D confers significant resistance to NNRTIs [12]. In another experiment, V108I emerged at an EFV concentration of 100 nmol/l and L100I at an EFV of 1000 nmol/l (Fig. 1b). Both L100I and V108I are listed in the International AIDS Society (IAS)-USA Resistance Table [20] as EFV resistance mutations. In the last experiment on HIV-1_{I135L}, G190A emerged followed by V106A (Fig. 1c). The latter two are also listed in the IAS-USA Table. In one of the three induction experiments on HIV-1_{I135V}, E138K emerged at an EFV of 100 nmol/l and L100I at an EFV of 1000 nmol/l (Fig. 1d). E138K is a rare mutation and not listed as a resistance mutation in the IAS-USA Table. It was reported that E138K alone did not alter drug susceptibility significantly, though it emerged during resistance induction experiments with ETR and other experimental NNRTIs (Brillant *et al.* 13th International HIV Drug Resistance Workshop, 2004; Su *et al.* 16th International HIV Drug Resistance Workshop, 2007) [21–23]. L100I emerged first followed by Y188H in another experiment, and L100I emerged first followed by V108I in the last experiment (Figure 1e and f). In one of the three induction experiments on HIV-1_{I135T}, V108I emerged at an EFV of 100 nmol/l and K101E at an EFV of 1000 nmol/l (Fig. 2a). In another experiment, V106I emerged first followed by V179D (Fig. 2b). The combination of V106I and V179D was confirmed to confer a significant NNRTI resistance by our group (unpublished data). In the last experiment, V108I emerged first followed by E138K and L100I (Fig. 2c). In one of the three induction experiments on HIV-1_{I135R}, L100I emerged at an EFV of 100 nmol/l followed

Table 1. Frequency of amino acids at codon 135 of HIV-1 reverse transcriptase in human leukocyte antigen-B*51-positive and human leukocyte antigen-B*51-negative patients.

135th amino acid	I	L	V	T	R
B*51 (+)/17 (%)	1 (5.9)	3 (17.6)	1 (5.9)	12 (70.6)	0 (0)
B*51 (−)/80 (%)	30 (37.5)	13 (16.3)	11 (13.8)	22 (27.5)	4 (5.0)
Total/97 (%)	31 (32.0)	16 (16.5)	12 (12.4)	34 (35.1)	4 (4.1)

HLA type was determined by standard sequence-based genotyping. HLA, human leukocyte antigen.

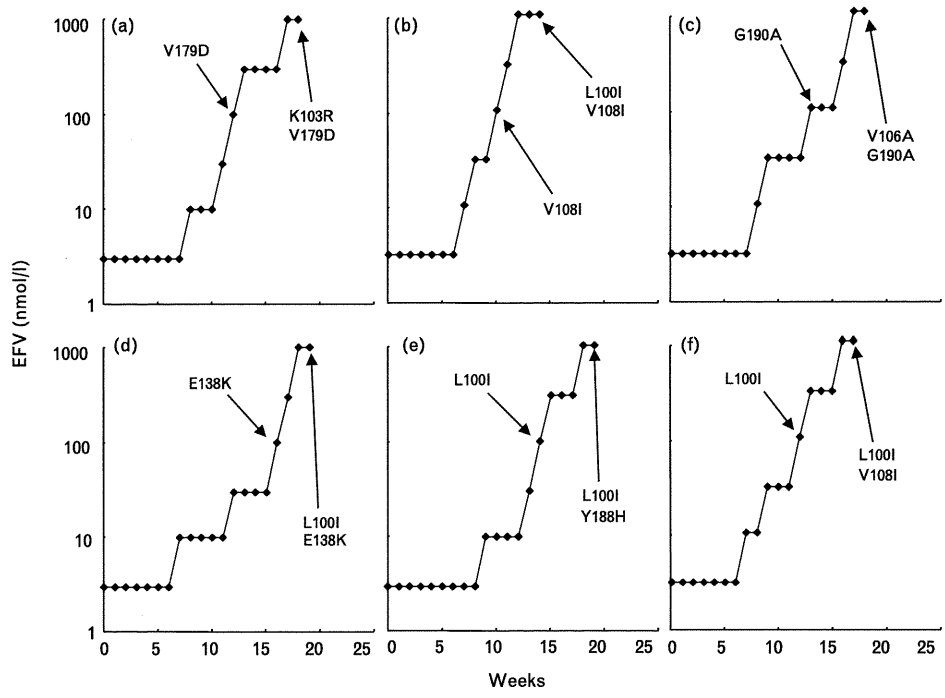

Fig. 1. Induction of efavirenz resistance from HIV-1_{I135L} and HIV-1_{I135V}. HIV-1_{I135L} (a–c) and HIV-1_{I135V} (d–f) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 *RT* gene in MT-2 cells. EFV, efavirenz; RT, reverse transcriptase.

by E138K at an EFV of 1000 nmol/l (Fig. 2d). In another experiment, E138K emerged first then G190A and V108I (Fig. 2e). In the last experiment, L100I emerged first followed by K101E (Fig. 2f). In summary, during the induction experiments, all the induced mutations were already known NNRTI-resistance mutations except for E138K, which emerged in one of the three induction experiments on HIV-1_{I135V}, in one of the three induction

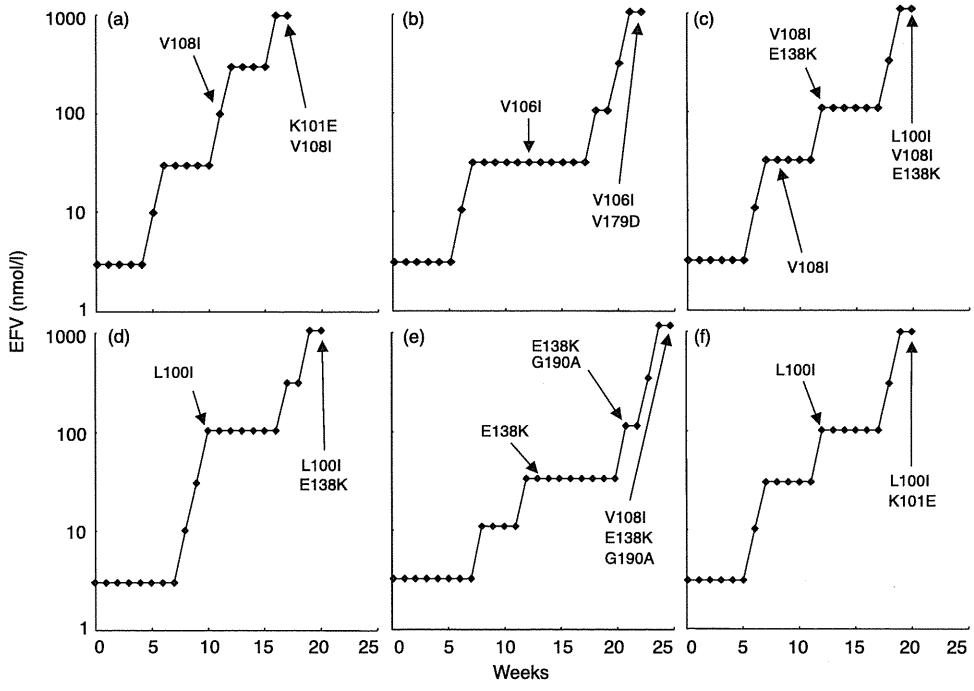

Fig. 2. Induction of efavirenz resistance from HIV-1_{I135T} and HIV-1_{I135R}. HIV-1_{I135T} (a–c) and HIV-1_{I135R} (d–f) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 *RT* gene in MT-2 cells. EFV, efavirenz; RT, reverse transcriptase.

experiments on HIV-1_{I135T}, and in two of the three induction experiments on HIV-1_{I135R}. We also performed EFV-resistance induction experiments on HIV-1_{WT} in triplicate using the same procedure. All the induced mutations were already known NNRTI-resistance mutations, whereas E138K did not emerge in any of the three induction experiments on HIV-1_{WT} (data not shown).

Nonnucleoside reverse transcriptase inhibitor resistance conferred by E138K combined with I135X

During the induction experiments on HIV-1s harboring I135X, the emergence of E138K, which is usually a rare mutation, was often observed. To analyze the effects of E138K alone and its combination with I135X on NNRTI susceptibility, a panel of recombinant HIV-1 clones was constructed and their IC_{50} values for EFV, NVP, and ETR were determined. As expected, I135X alone did not confer significant NNRTI resistance (Table 2). The combination of I135T and E138K (I135T/E138K) conferred significant resistance to EFV, NVP, and ETR, though E138K alone did not change NNRTI susceptibility as reported previously (Su *et al.* 16th International HIV Drug Resistance Workshop, 2007) [22,23]. I135L/E138K and I135R/E138K conferred significant resistance to NVP and ETR, respectively. In summary, E138K conferred significant resistance when combined with some of the I135X mutations, especially I135T, which is the most prevalent in treatment-naive individuals in Japan (Table 1).

Structural modeling of reverse transcriptase harboring I135T and E138K

The in-vitro drug susceptibility assay described above showed that I135T/E138K conferred the most efficient resistance to EFV and significant resistance to NVP and ETR. To analyze the molecular mechanisms by which E138K combined with I135T alter NNRTI susceptibility, we conducted a structural analysis that included computational methods. A total of 12 structural models of reverse transcriptase-NNRTI complexes were constructed with four reverse transcriptases (wild-type, I135T, E138K, and I135T/E138K) and three NNRTIs (EFV, NVP, and ETR). We first calculated the binding energies between reverse transcriptase and NNRTI. Differences in the binding energies between mutant and wild-type complexes ($\Delta\Delta$Gb) were calculated using the models. The $\Delta\Delta$Gb value correlated positively with the logarithm of fold resistance value obtained by our in-vitro drug susceptibility assay described above: a greater reduction in the binding energy correlated with a greater resistance ($r=0.77$, $P<0.02$) [24], suggesting that our modeling appropriately reflects the actual binding mode between the reverse transcriptase molecule and NNRTI. In the 12 models tested, the $\Delta\Delta$Gb value of the I135T/E138K RT-NNRTI complex was persistently larger than wild-type and single mutation reverse transcriptases, indicating that I135T/E138K caused a larger loss of interactions between reverse transcriptase and NNRTI than the single mutations. We then examined the structural changes in the loss of interactions by I135T/E138K. In the wild-type reverse transcriptase, the E138 positioned relatively closely to the EFV, which could contribute to the generation of van der Waals and electrostatic interactions between reverse transcriptase and NNRTI (Fig. 3a). The I135T single substitution caused no significant changes in the steric position of the E138 side chain (Fig. 3b). E138K substitution caused significant changes in the steric position of the E138 side chain (Fig. 3c), whereas the calculated van der Waals energy was similar to that of wild-type reverse transcriptase. I135T/E138K also caused significant changes in the steric position of the K138 side chain, but the orientation of the side chain was different from that of the E138K single mutant reverse transcriptase, possibly due to the interactions between T135 and K138 (Fig. 3d). The K138 conformation in the $RT_{I135T/E138K}$ generated a steric gap between K138 and EFV, and significantly reduced van der Waals energy. In addition, the conformational change necessitated increased electrostatic energy of the reverse transcriptase–EFV complex. These data suggest that an appropriate steric position of the 138th residue is critical for the generation

Table 2. Nonnucleoside reverse transcriptase inhibitor susceptibility of recombinant HIV-1 sequences.

	Mean IC_{50} (μmol/l) ± SD (fold resistance*)		
HIV-1	EFV	NVP	ETR
Wild-type	0.002 ± 0.0007	0.05 ± 0.01	0.0012 ± 0
I135L	0.003 ± 0.0005 (1.5)	0.07 ± 0.01 (1.4)	0.0012 ± 0.0002 (1)
I135V	0.0024 ± 0.0003 (1.2)	0.04 ± 0.01 (0.8)	0.0011 ± 0.0001 (0.9)
I135T	0.002 ± 0.001 (1)	0.06 ± 0.01 (1.2)	0.0016 ± 0.0002 (1.3)
I135R	0.003 ± 0.001 (1.5)	0.03 ± 0.01 (0.6)	0.0012 ± 0.0002 (1)
E138K	0.004 ± 0.0004 (2)	0.08 ± 0.01 (1.6)	0.0026 ± 0.0001 (2.2)
I135L/E138K	0.003 ± 0.001 (1.5)	0.23 ± 0.02 (4.6)	0.0033 ± 0.0006 (2.8)
I135V/E138K	0.006 ± 0.001 (3)	0.04 ± 0.01 (0.8)	0.0033 ± 0.0006 (2.8)
I135T/E138K	0.014 ± 0.002 (7)	0.19 ± 0.06 (3.8)	0.005 ± 0.0002 (4.2)
I135R/E138K	0.005 ± 0.002 (2.5)	0.14 ± 0.04 (2.8)	0.0047 ± 0.0003 (3.9)

The drug susceptibility assay was performed in triplicate and repeated three times. EFV, efavirenz; ETR, etravirine; NVP, nevirapine.
*Fold resistance was calculated by comparing viral IC_{50} with that of wild-type HIV-1.

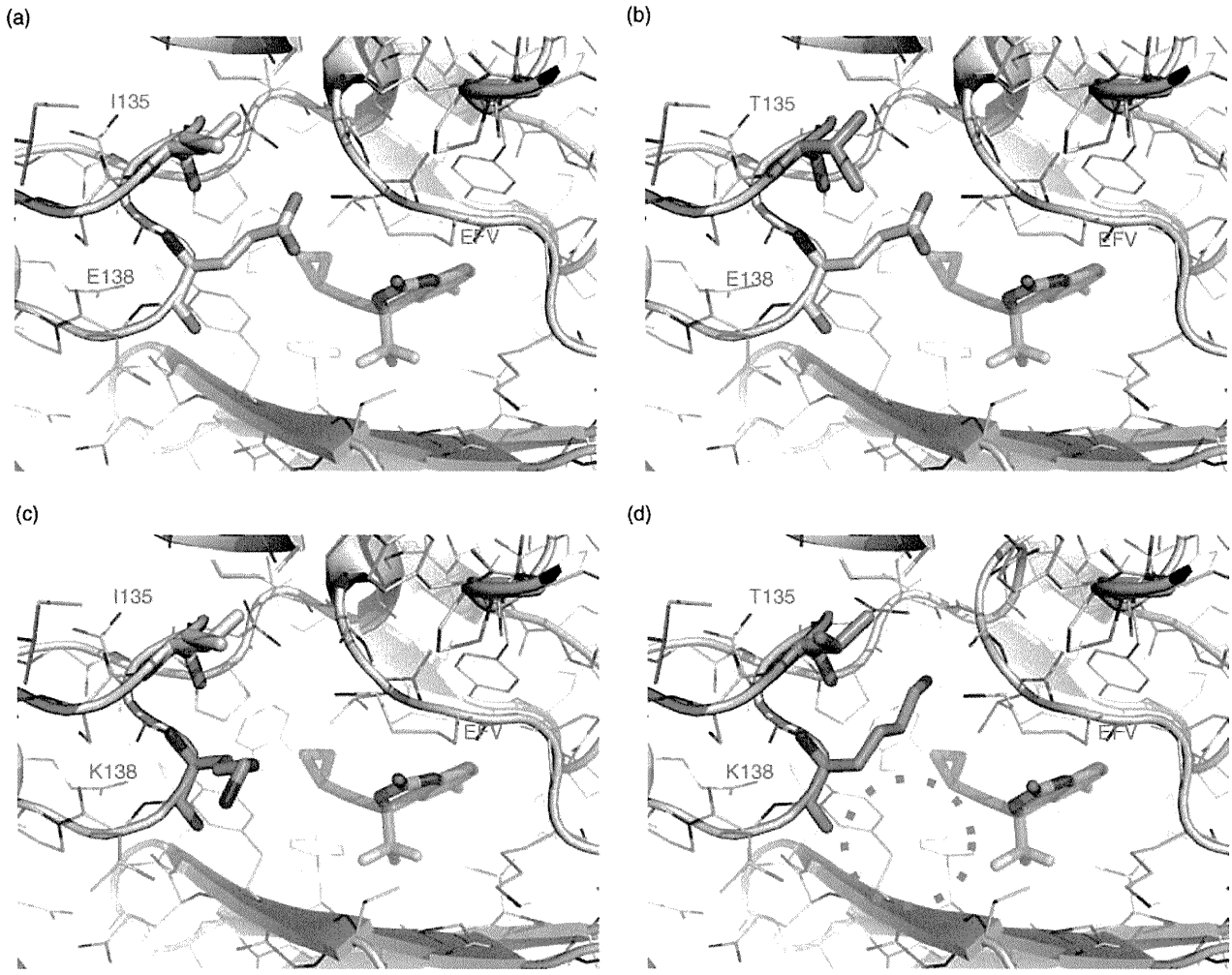

Fig. 3. Structural models of HIV-1 reverse transcriptase–efavirenz complexes. The binding clefts of four complex models are shown. (a) $RT_{wild\text{-}type}$, (b) RT_{I135T}, (c) RT_{E138K} and (d) $RT_{I135T/E138K}$. Sticks indicate the amino acids at positions 135 and 138 of RT, and the atoms of EFV. The mutated residues (I135T and E138K) and the EFV atoms are highlighted with orange and cyan sticks, respectively. The dotted circle in panel D indicated the enlarged gap by I135T/E138K mutations. EFV, efavirenz; RT, reverse transcriptase.

of an optimal EFV binding pocket, and that I135T/E138K, but not the single mutations, effectively break the binding pocket for EFV.

Discussion

As the HIV-1 pandemic progresses, viral genetic diversity is increasing and becoming geographically heterogeneous [25,26]. We recently indicated that HIV-1 adapts to CTL by acquiring escape mutations in the CTL epitopes, and that such escape variants are increasing in the populations at an alarming high rate of corresponding HLA alleles [4]. When escape mutations occur in drug target proteins, they may alter the mutation patterns of drug resistance even if they do not confer drug resistance themselves. In this study, we focused on I135X in reverse transcriptase, which are escape mutations of HLA-B*51-restricted CTL, because I135X are the prevailing mutations and accumulating in Japan, where the frequency of HLA-B*51 is high (~20%). Cultural passages of HIV-1 sequences harboring I135X in the presence of increasing concentrations of EFV induced the emergence of E138K, which is not listed as a resistance mutation in the IAS-USA Table. The analysis of recombinant HIV-1 sequences showed that the combination of E138K and some of the I135X, especially I135T, which is most frequent, conferred significant resistance to NNRTI, though solo E138K did not alter drug susceptibility significantly. However, E138K did not always emerge in triplicate experiments of EFV-resistance induction from HIV-1 sequences harboring I135X, whereas the already known NNRTI-resistance mutations emerged. Importantly, variable mutation patterns emerged under the same conditions of resistance induction experiments,

indicating that the drug selective pressure is one of the driving forces making the genetic diversity of HIV-1 at population levels as CTL pressure does (HLA-B*51-restricted CTL pressure selects not only I135T but also other I135Xs).

In clinical data, Richard *et al.* [27] examined HIV-1 reverse transcriptase sequences in treated Ugandans. In their longitudinal cohort, the HIV-1 infecting one patient (JLT05) acquired I135T/E138K during EFV-containing treatment without any other NNRTI resistance-associated mutations (GenBank: AY556834). Marconi *et al.* [28] performed genotypic resistance testing in patients who experienced virologic failure during their first antiretroviral therapy, and the HIV-1 in one patient (SW065) was found to have I135T/E138K after the failure of EFV-containing treatment (EU308076). In tipranavir clinical trials, the HIV-1s of seven cases who experienced NNRTI treatment failure harbored I135T/E138K (DQ880123, DQ880358, DQ879290, DQ880378, DQ877823, DQ878145, and DQ878874) [29]. These data indicate that I135T/E138K confers significant NNRTI resistance *in vivo* also, suggesting that HLA-B*51-restricted pressure may alter the mutation patterns of NNRTI resistance by inducing escape mutations.

Evidences for the interactions between CTL and drug resistance mutations are accumulating [30–34]. Considering that HIV-1 adapts to particular human HLA alleles and evolves among infected individuals, drug mutation patterns may be affected and altered in currently prevailing viruses. Analysis of drug resistance mutations and development of new antiretroviral agents against laboratory HIV-1 strains derived from isolates obtained decades ago may not always be a suitable strategy. The use of recently obtained clinical isolates may be critical and indispensable in some studies.

Acknowledgements

This work was supported in part by a Grant-in Aid for AIDS research from the Ministry of Health, Labor, and Welfare (H20-AIDS-002), and the Global Center of Excellence Program (Global Education and Research Center Aiming at the Control of AIDS) from the Ministry of Education, Science, Sports and Culture of Japan.

H.G. designed and executed the study, analyzed the data and wrote the manuscript. H.O. and H.S. performed computational analysis and wrote the manuscript. A.H. and T.H. executed the study and collected data. M.T. provided the hypothesis and participated in discussion and review. S.O. participated in discussion and review and supervised the study.

There are no conflicts of interest.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2010, p. 1596–1602 Vol. 54, No. 4
0066-4804/10/$12.00 doi:10.1128/AAC.01480-09

Combination of V106I and V179D Polymorphic Mutations in Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confers Resistance to Efavirenz and Nevirapine but Not Etravirine▽†

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Received 19 October 2009/Returned for modification 16 December 2009/Accepted 22 January 2010

Etravirine (ETV) is a second-generation nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) introduced recently for salvage antiretroviral treatment after the emergence of NNRTI-resistant human immunodeficiency virus type 1 (HIV-1). Following its introduction, two naturally occurring mutations in HIV-1 RT, V106I and V179D, were listed as ETV resistance-associated mutations. However, the effect of these mutations on the development of NNRTI resistance has not been analyzed yet. To select highly NNRTI-resistant HIV-1 *in vitro*, monoclonal HIV-1 strains harboring V106I and V179D (HIV-1_{V106I} and HIV-1_{V179D}) were propagated in the presence of increasing concentrations of efavirenz (EFV). Interestingly, V179D emerged in one of three selection experiments from HIV-1_{V106I} and V106I emerged in two of three experiments from HIV-1_{V179D}. Analysis of recombinant HIV-1 clones showed that the combination of V106I and V179D conferred significant resistance to EFV and nevirapine (NVP) but not to ETV. Structural analysis indicated that ETV can overcome the repulsive interactions caused by the combination of V106I and V179D through fine-tuning of its binding module to RT facilitated by its plastic structure, whereas EFV and NVP cannot because of their rigid structures. Analysis of clinical isolates showed comparable drug susceptibilities, and the same combination of mutations was found in some database patients who experienced virologic NNRTI-based treatment failure. The combination of V106I and V179D is a newly identified NNRTI resistance pattern of mutations. The combination of polymorphic and minor resistance-associated mutations should be interpreted carefully.

Human immunodeficiency virus type 1 (HIV-1) sequences differ among infected individuals, and there are a number of naturally occurring amino acid changes commonly found in treatment-naïve patients (3, 23, 28). These polymorphic changes can occur even in genes that encode drug target proteins, and in fact, some drug resistance-associated mutations in protease genes are often present in treatment-naïve individuals, especially in non-subtype B clade-infected individuals (13, 15, 22). Minor resistance mutations, which are considered to compensate for the impaired replication fitness of viruses containing major resistance mutations, do not have a substantial effect on the viral phenotype by themselves (14, 27). In the reverse transcriptase (RT) coding region, drug resistance-associated mutations were detected at a low frequency in treatment-naïve individuals regardless of the HIV-1 clade. However, etravirine (ETV), a second-generation nonnucleoside RT inhibitor (NNRTI), has been available in the clinical setting and the following have been listed as ETV resistance-associated mutations in RT: V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190S/A, and M230L (14). V106I and V179D are often found in treatment-naïve individuals but are considered to have no substantial impact on NNRTI-containing treatment by themselves. ETV exhibits activity against many viruses that are resistant to first-line NNRTIs, including efavirenz (EFV) and nevirapine (NVP), and shows clinical efficacy in salvage treatment after NNRTI treatment failure (18, 21). However, it is possible that EFV- and NVP-resistant viruses derived from HIV-1 harboring V106I or V179D could compromise the efficacy of ETV. To determine the impact of these polymorphic mutations on the mutation patterns of NNRTI resistance, EFV-resistant HIV-1 strains were selected *in vitro* from monoclonal viruses harboring V106I and V179D, respectively. The virologic effects of selected specific mutation patterns were analyzed by constructing recombinant HIV-1 clones, and their clinical relevance was confirmed by analysis of isolates from infected individuals.

MATERIALS AND METHODS

HIV-1 sequences and clinical isolates from treatment-naïve individuals. HIV-1 RT sequences were analyzed in 364 antiretroviral treatment-naïve infected individuals who visited the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, in 2007 and 2008 and gave written informed consent to this study. Viral RNA was extracted from stocked plasma samples, and the HIV-1 RT coding region was amplified by RT-PCR and nested PCR using previously published primer pairs (7, 10, 11). Direct sequencing was performed using dye terminators, and the HIV-1 subtypes of the sequences obtained were determined by the neighbor-joining method. Clinical HIV-1 isolates were obtained using MAGIC-5 cells (CCR5- and CD4-expressing HeLa-LTR-β-D-gal cells) from fresh plasma samples collected from seven of the above-mentioned treatment-naïve patients and stored at −80°C until use (9).

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 1 February 2010.

TABLE 1. Frequencies of amino acids at positions associated with NNRTI resistance mutations in HIV-1 RT in treatment-naïve patients

Position	Amino acid, frequency [*n* (%)/364][a]					
90	V, 361 (99.2)	I, 3 (0.8)				
98	A, 344 (94.5)	S, 20 (5.5)				
101	K, 352 (96.7)	Q, 8 (2.2)	R, 3 (0.8)	E, 1 (0.3)		
103	K, 353 (97.0)	R, 7 (1.9)	N, 2 (0.5)	Q, 2 (0.5)		
106	V, 355 (97.5)	I, 9[b] (2.5)				
108	V, 362 (99.5)	I, 2 (0.5)				
138	E, 361 (99.2)	K, 1 (0.3)	A, 1 (0.3)	G, 1 (0.3)		
179	V, 312 (85.7)	D, 21[c] (5.8)	I, 21 (5.8)	E, 4 (1.1)	A, 4 (1.1)	T, 1 (0.3) N, 1 (0.3)

[a] Only wild-type amino acids (L, Y, Y, G, P, and M, respectively) were identified at the 100th, 181st, 188th, 190th, 225th, and 230th positions of HIV-1 RT.
[b] Including two cases with V90/A98/K101Q/K103/V106I/V108/E138/V179, two cases with V90/A98/K101/K103/V106I/V108/E138/V179I, one case with V90/A98/K101/K103/V106I/V108/E138/V179D, and four cases with V90/A98/K101/K103/V106I/V108/E138/V179.
[c] Including 2 cases with V90/A98S/K101/K103/V106/V108/E138/V179D, 1 case with V90/A98/K101E/K103R/V106/V108/E138/V179D, 1 case with V90/A98/K101/K103R/V106/V108/E138/V179D, 1 case with V90/A98/K101/K103/V106I/V108/E138/V179D, and 16 cases with V90/A98/K101/K103/V106/V108/E138/V179D.

Generation of recombinant HIV-1 strains. The desired mutations were introduced into the XmaI-NheI region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (10, 16). The XmaI-NheI fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1 strain BH 10. pNL_{H219Q} harbors the H219Q mutation in the HIV-1 Gag region, which facilitated HIV-1 replication in MT-2 and H9 cells (6, 8). HIV-1 derived from pNL_{H219Q} was used as the wild type. Determination of the nucleotide sequences of the plasmids confirmed that each clone had the desired mutations but was devoid of unintended mutations. Each molecular clone was transfected into COS-7 cells with the GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA), and the virions obtained were harvested 48 h after transfection and stored at −80°C until use.

Selection of EFV-resistant HIV-1. The infectious HIV-1 clones harboring the V106I ($HIV\text{-}1_{V106I}$) and V179D ($HIV\text{-}1_{V179D}$) mutations in their RTs were propagated in MT-2 cells in the presence of increasing concentrations of EFV (7, 8, 31). Briefly, MT-2 cells (1×10^5) were exposed to 500 blue-cell-forming units (BFU) in MAGIC-5 cells containing $HIV\text{-}1_{V106I}$ and $HIV\text{-}1_{V179D}$ and cultured in the presence of EFV at an initial concentration of 3 nM. Viral replication was monitored by observation of the cytopathic effect in MT-2 cells. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased by 0.5-log-fold. This selection was carried out for a total of 14 passages. The proviral HIV-1 RT coding region in infected MT-2 cells was amplified and sequenced at several passages.

Drug susceptibility assay. EFV, NVP, and ETV were generously provided by Merck Co., Inc. (Rahway, NJ), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Tibotec Pharmaceuticals (Little Island, Co., Cork, Ireland), respectively. Recombinant and isolated HIV-1 susceptibility to EFV, NVP, and ETV was determined in triplicate using MAGIC-5 cells (10, 12, 16). Briefly, MAGIC-5 cells were infected with an adjusted virus stock (300 BFU) in various concentrations of NNRTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Takara Shuzo, Ohtsu, Japan). The blue-stained cells were counted under a light microscope. The drug concentrations that inhibited 50% of the stained cells of a drug-free control (EC_{50}s) were determined by referring to the dose-response curve. The drug susceptibility assay was performed in triplicate and repeated three times. Fold resistance was calculated by comparing the viral EC_{50} with that of monoclonal wild-type HIV-1 ($HIV\text{-}1_{WT}$). Drug resistance was judged significant when it was higher than threefold.

Viral replication kinetics assay. MT-2 cells (1×10^5) were exposed to each infectious virus preparation (500 BFU) for 2 h, washed twice with phosphate-buffered saline (PBS), and cultured in the presence or absence of 10 nM EFV, 100 nM NVP, or 10 nM ETV. The culture supernatants were harvested every other day, and p24 Gag amounts were determined with a chemiluminescence enzyme immunoassay kit (Fuji-Rebio, Tokyo, Japan). Replication assays were performed in triplicate and repeated three times using independently generated virus preparations (7).

Competitive HIV-1 replication assay. Freshly prepared H9 cells (3×10^5) were exposed to mixtures of paired virus preparations (300 BFU each) to be examined for their replication ability for 2 h, washed twice with phosphate-buffered saline (PBS), and cultured in the absence or presence of 10 nM EFV or 100 nM NVP as described previously (7, 17). On day 1, one-third of the infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 week). Every 7 days, the supernatant of the virus culture was transferred to new uninfected H9 cells; the cells harvested at the end of each passage were subjected to direct DNA sequencing of the HIV-1 RT coding region, and the change in the viral population was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed at the end of the assay.

Structure modeling. We constructed 18 structural models of the HIV-1 RT and NNRTI complex by computational analysis. First, we constructed the initial models of wild-type RT with one of three NNRTIs by homology modeling using Molecular Operating Environment 2007.09.02 (Chemical Computing Group, Montreal, Quebec, Canada). The crystal structures of RT with NNRTIs (Protein Data Bank codes 1IKW [20], 1VRT [26], and 1SV5 [4]) were used as template structures. The homology modeling enabled the building of missing atoms in template structures. The ff94 force field and distance-dependent electrostatic energy function were applied in the modeling. Next, we refined the initial models by energy minimization using the sander module of the AMBER9 software package in two steps. In the first step, energies for the NNRTIs in the complex models were minimized in the gas phase by the conjugated gradient method. When the energy was not converged until 10,000 steps, this step was ignored. In the second step, energies of whole structures were converged up to 0.5 kcal/mol/Å by 50 steps of the steepest-descent method and the subsequent conjugated gradient method under implicit water solvent conditions. In each minimization, the AMBER ff03 (5, 19), the general AMBER force field (30), and the generalized Born implicit solvent surface area method (IGB = 2) (24) were applied for potential energy calculations. The cutoff for long-distance interaction energy was set at 15.0 Å. The charge and type of every atom in NNRTIs were automatically assigned using the AMBER9 Antechamber module. We also constructed five mutant RTs with the NNRTIs by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL ver. 0.99rc6 (http://www.pymol.org). V106A, V106I, and V179D mutants had one, three to five, and five possible conformers, respectively. The structural model of each conformer was refined by a method similar to that used in the wild-type models. Among the refined conformers, we selected the conformer with the lowest energy as each mutant model.

RESULTS

Frequencies of NNRTI resistance mutations in treatment-naïve individuals. To determine the frequency of NNRTI resistance-associated mutations, the HIV-1 RT coding region was analyzed and the viral subtype was determined in 364 treatment-naïve infected individuals. The most frequent subtype was clade B (*n* = 334; 91.8%), followed by clade AE (*n* = 20; 5.5%). Clades C and G were also found and at similar low frequencies (*n* = 5; 1.4%). Variable amino acid substitutions were identified at the positions of NNRTI resistance-associated mutations, including the 90th, 98th, 101st, 103rd, 106th, 108th, 138th, and 179th, though only the wild-type amino acids were observed at the 100th, 181st, 188th, 190th, 225th, and 230th positions of HIV-1 RT (Table 1). K103N and V108I are

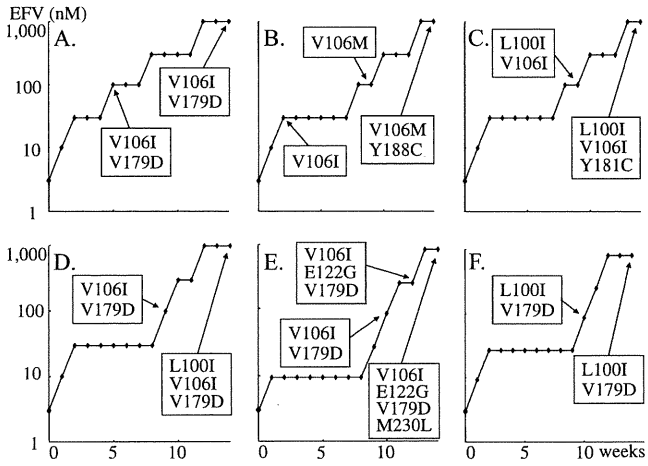

FIG. 1. Selected amino acid substitutions under selective pressure from EFV. HIV-1_{V106I} (A to C) and HIV-1_{V179D} (D to F) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The selected amino acid substitutions were analyzed at several passages by sequencing the proviral 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.

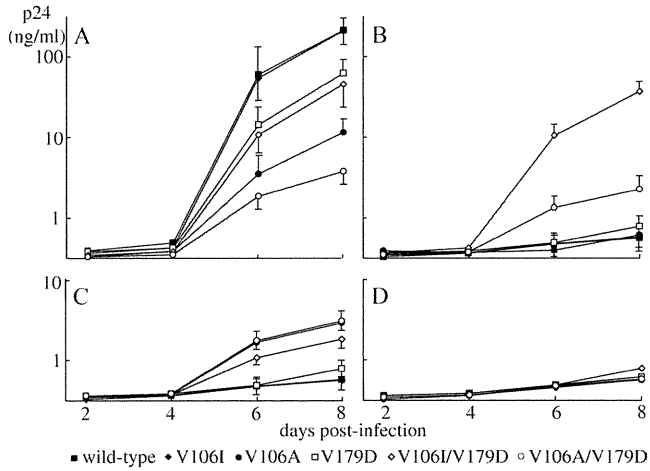

FIG. 2. Replication kinetics of recombinant HIV-1 clones in the absence and presence of NNRTIs. Recombinant HIV-1 clones were propagated in MT-2 cells in the absence (A) and presence of 10 nM EFV (B), 100 nM NVP (C), or 10 nM ETV (D). The concentration of p24 in the culture medium was measured every 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.

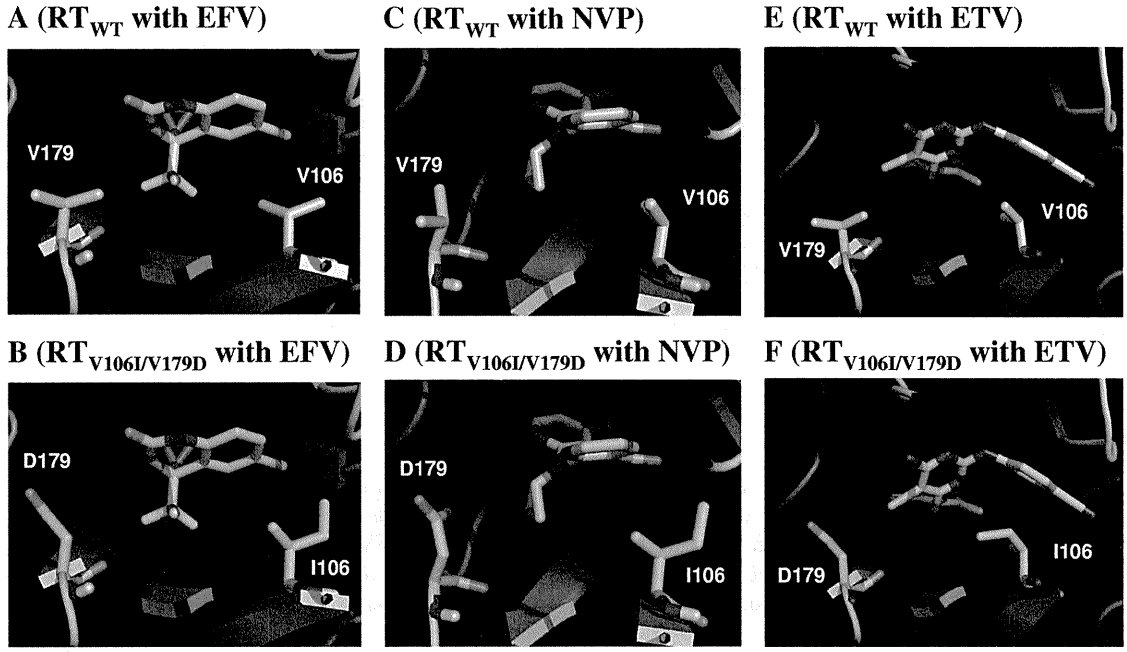

FIG. 3. Interactions of NNRTIs with the 106th and 179th residues of RTs. Interaction sites of NNRTIs and RTs in the models are shown. (A) Wild-type RT (RT_{WT}) with EFV. (B) $RT_{V106I/V179D}$ with EFV. (C) RT_{WT} with NVP. (D) $RT_{V106I/V179D}$ with NVP. (E) RT_{WT} with ETV. (F) $RT_{V106I/V179D}$ 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