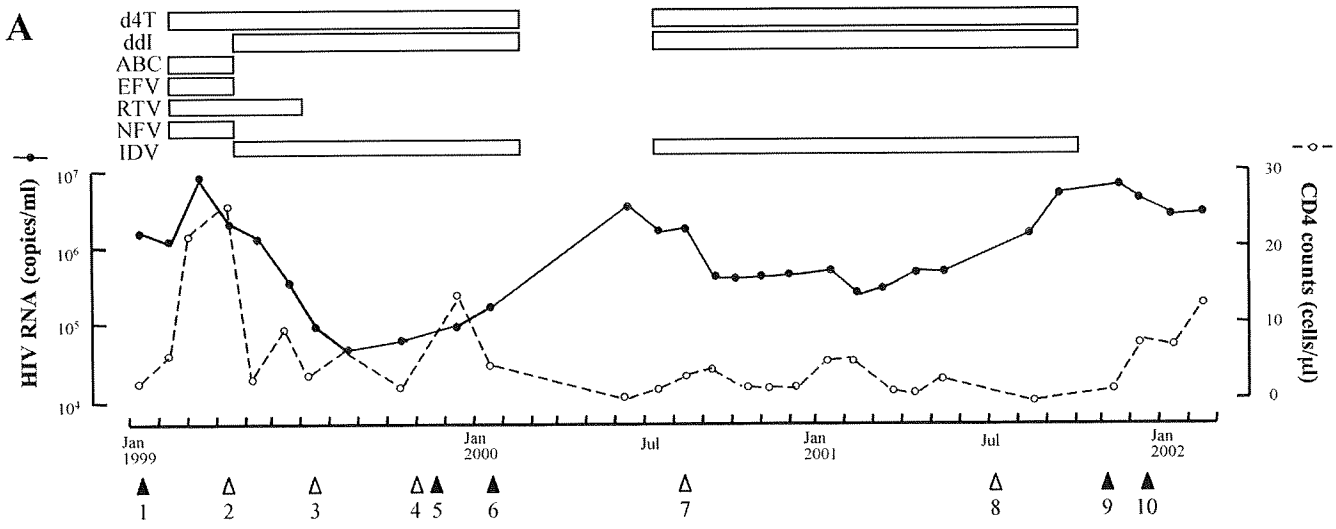

B

Time point	Resistance-associated and unique mutations											Fold increase in EC_{50}								
	M41	D67	T69	K70	V75	V118	L210	T215	L283	N348	I393	AZT	ddI	ddC	d4T	3TC	ABC	NVP	DLV	EFV
1	-	-	-	-	-	-	-	-	I	-	L	2	1.5	0.6	1.0	0.9	0.8	2.2	1	1
2	-	-	-	-	-	-	-	-	I	-	L					N.D.				
3	M/L	-	-	-	-	-	-	-	I	-	L					N.D.				
4	L	N	-	-	M	-	W	Y	I	N/I	L					N.D.				
5	L	N	-	-	M	-	W	Y	I	I	L	**44**	**4.3**	0.6	1.8	1.6	1.6	**44**	**8.5**	**3**
6	L	N	-	-	M	I	W	Y	I	I	L	**275**	**5.9**	2.5	**3.2**	**3.4**	2	**25**	**12**	1
7	-	-	-	-	-	-	-	-	I	-	L					N.D.				
8	L	T/N	-/SSG	E	M	I	W	Y	I	N/I	L					N.D.				
9	-	-	-	-	V/M	V/I	L/W	T/Y	I	-	-	2	1.2	0.6	1.1	0.6	1	2.4	1.5	0.7
10	-	-	-	-	-	-	-	-	I	-	-	2.8	0.7	0.6	1	0.8	1.2	1.8	1	1

FIG. 1. The course of patient and drug resistance profiles of clinical isolates obtained from the patient. (A) The drug treatment history is indicated at the top of the graph. The virologic responses represented by plasma viral load and CD4 counts of peripheral blood are shown. Open triangles indicate the time points of genotypic assays. Closed triangles indicate the time points of isolation of clinical isolates for genotypic assays (also see Fig. S1 in the supplemental material) and phenotypic assays (also see Table S1 in the supplemental material showing actual EC_{50} values as mean values and standard deviations from three independent experiments). From February to June 2000 and after October 2001, the chemotherapy was interrupted due to severe adverse effects. (B) The viruses acquired NRTI resistance mutations sequentially as shown. Susceptibility to compounds tested in at least three independent experiments is shown as the relative increase in the EC_{50} compared to HIV-1_{WT} obtained from a pNL4-3-based plasmid. An increase larger than 3.0-fold is indicated in bold. NRTI or NNRTI resistance mutations were reported in the HIV drug resistance database maintained by International AIDS Society 2006, the Stanford University (Stanford, CA) and Los Alamos National Laboratory (Los Alamos, NM), http://hivdb.stanford.edu/and http://resdb.lanl.gov/Resist_DB/, respectively. RTV, ritonavir; NFV, nelfinavir; IDV, indinavir.

(8.5- and 12-fold, respectively) but lacked any known NNRTI resistance-associated mutations except for L283I, which influences susceptibility of NNRTIs when combined with I135L/M/T (6) (Fig. 1B). However, L283I was detected at all points without I135L/M/T even in phenotypically sensitive viruses; therefore, it is unlikely that this single mutation is involved in the resistance. After the interruption at time points 9 and 10, the majority of HIV-1 detected in the plasma reverted to WT and was susceptible to all RTIs tested. The patient was previously treated with a regimen containing EFV, not NVP, for several months prior to the appearance of the N348I mutation. Importantly, this mutation was not detected in genotypic assays during treatment with EFV, but it was first detected 6 months after removal of EFV and use of ddI in the following regimen. Phenotypic and genotypic information at time point 5 shows that resistance to NVP and DLV was present while the patient was on a regimen that did not include any NNRTIs and in the absence of any known NNRTI resistance-related mutations. Thus, it is unlikely that the phenotypically identified NNRTI resistance in the patient was induced by the previous EFV-containing therapy.

RT C-terminal region confers NVP resistance. To identify the mutation(s) responsible for the resistance to NVP and DLV, we constructed chimeric clones with cDNA fragments of the RT region derived from the clinical isolates. Briefly, the N-terminal (amino acids 15 to 267) and C-terminal (amino acids 268 to 560) RT coding regions of clinical isolates were PCR amplified separately and used for replacement of the corresponding regions in the WT sequence of pNL-RT_{WT}. These chimeric clones were then examined for their susceptibility to RTIs (Table 1). Only the clones containing the C-terminal region derived from CL-6 isolated at time point 6 and

TABLE 1. Susceptibility of chimeric HIV-1 clones with N- and/or C-terminal RT region substitutions

RT-replaced region		EC_{50} (fold increase)[a]				
N terminus[b]	C terminus[c]	AZT	ddI	NVP	DLV	EFV
WT[d]	WT	0.038 ± 0.012	2.6 ± 1.04	0.05 ± 0.01	0.03 ± 0.01	0.003 ± 0.001
CL-6[e]	CL-6[e]	3.37 ± 0.97 **(89)**	14.3 ± 0.58 **(5.5)**	1.2 ± 0.21 **(24)**	0.16 ± 0.02 **(5.3)**	0.007 ± 0.004 (2.3)
CL-9[f]	CL-9[f]	0.04 ± 0.01 (1.1)	2.3 ± 1.21 (0.9)	0.13 ± 0.07 (2.6)	0.06 ± 0.02 (2)	0.004 ± 0.002 (1.3)
CL-6	WT	1.24 ± 0.34 **(33)**	4.6 ± 1.50 (1.8)	0.12 ± 0.06 (2)	0.04 ± 0.02 (1.3)	0.002 ± 0.001 (0.7)
WT	CL-6	0.19 ± 0.04 **(5)**	13.7 ± 2.31 **(5.3)**	1.67 ± 0.23 **(33)**	0.39 ± 0.06 **(13)**	0.006 ± 0.002 (2)
CL-6	CL-9	1.50 ± 0.95 **(39)**	5.9 ± 1.21 (2.3)	0.10 ± 0.05 (2)	0.04 ± 0.02 (1.3)	0.002 ± 0.001 (0.7)

[a] The data shown are mean values ± standard deviations obtained from the results of at least three independent experiments, and the relative increase in the EC_{50} values for recombinant viruses compared with WT is shown in parentheses. Bold indicates an increase in EC_{50} value greater than threefold relative to the WT.
[b] RT N-terminal region contains mainly the domains of finger and palm and partially thumb (amino acid positions 15 to 267).
[c] RT C-terminal region contains domains of thumb, connection, and RNase H (amino acid positions 268 to 560).
[d] DNA fragment is identical to pNL-RT_{WT}.
[e] N- and C-terminal regions of CL-6 contained T39A/M41L/K43E/D67N/V75M/V118I/I132V/L210W/T215Y and N348I/I393L in their coding regions, respectively (see also Fig. S1 in the supplemental material).
[f] No resistance-associated mutations were observed in either the N- or C-terminal region of CL-9 (also see Fig. S1 in the supplemental material).

showed resistance (Fig. 1; see also Fig. S1 in the supplemental material) to NVP and DLV. Interestingly, the C-terminal region also conferred resistance to AZT and ddI even in the absence of AZT resistance mutations that normally reside at the N-terminal region within amino acids 41 to 219. Recently, mutations in the connection subdomain, including G335D, N348I, and A360T, have been shown to confer AZT resistance (28). In these clinical isolates the C-terminal region contained four unique mutations in the connection subdomain: G335D, N348I, A360T, and I393L (see Fig. S1 in the supplemental material). G335D and A360T were continuously observed at every time point and are polymorphisms related to subtype D. Since these isolates showed no phenotypic resistance (Table 1 and Fig. 1B), it is unlikely that G335D and A360T are involved in the resistance, at least in subtype D. I393L was also continuously detected from time point 1 but disappeared after the treatment interruption at time point 9 (Fig. 1) while N348I appeared only from time points 4 to 6 and at point 8 under treatment.

To further clarify the effect of mutations at residues 348 and 393 on drug resistance, we generated the N348I and/or I393L mutations in the C-terminal region by site-directed mutagenesis on a pNL-RT_{WT} background. Consistent with the phenotypic experiments and the experiments with chimeric viruses, we found that the N348I substitution conferred resistance to AZT, ddI, NVP, and DLV. In contrast, we found that the I393L mutation caused no significant resistance by itself (Table 2). Furthermore, the combination of I393L with N348I did not show any significant increase in NVP resistance compared to N348I alone.

To address whether N348I further increases the level of AZT resistance in the presence of TAMs, we examined the effect of N348I on AZT susceptibility in the presence or absence of the classical AZT resistance mutations M41L/T215Y. M41L/T215Y or N348I showed only moderate resistance to AZT whereas a combination of M41L/T215Y and N348I further enhanced AZT resistance (Table 2). These data demonstrate that the N348I mutation is responsible for this cross-resistance to multiple members of the NRTI and NNRTI families and enhances AZT resistance induced by TAMs.

Viral replication kinetics. Since N348I and I393L immediately disappeared after cessation of HAART, we examined whether these mutations have an effect on viral replication kinetics using the p24 antigen production assay and a competitive HIV-1 replication assay (CHRA). In the p24 antigen production assay, acquisition of N348I drastically impaired replication in MT-2 and SupT1 cells (Fig. 2A and B). However, a moderately low reduction of replication kinetics was observed in PM1, H9 cells, and PHA-stimulated PBMCs (Fig. 2C, D, and E). HIV-1 carrying the mutation I393L (HIV-1_{I393L}) showed comparable replication kinetics in all cells tested. A combination of I393L with N348I showed no apparent change of replication kinetics in MT-2, SupT1 cells, and PHA-stimulated PBMCs (Fig. 2A, B, and E) and reduction in PM1 cells (Fig. 2C) compared to N348I alone. CHRA was performed for further comparison of replication kinetics in H9 cells. During 6 weeks in culture, we observed little difference in viral replication in H9 cells (Fig. 2F). A lack of an effect of I393L on the replication of N348I was confirmed by CHRA (Fig. 2G). These results indicate that N348I impairs viral replication in a cell-type-dependent manner and that I393L exerts little effect on viral replication of either the WT or N348I clones. Thus, I393L appears to be one of the specific polymorphisms for this isolate.

Insertion at 69 and N348I. At time point 8 we detected the transient presence of the fingers insertion mutation, a 2-amino-acid insertion at codon 69 in the presence of TAMs known to confer resistance to NRTIs by enhancing the excision reaction (3) (Fig. 1). Interestingly, at time point 8 WT N348 coexisted with resistant I348. To address whether these two MDR mutations were introduced onto the same RNA genome, we carried out clonal sequence analysis of PCR products. The results show that the fingers insertion and the N348I mutations were randomly introduced; seven, three, one, and six clones ($n = 17$) contained both mutations, the fingers insertion only, N348I only, and no mutation or insertion, respectively, in the background of TAMs (Table 3). In previous studies the fingers insertion complex emerged with the K70E mutation that was selected in vitro with adefovir (8) and β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (18), and it conferred low level resistance to TDF, ABC, and 3TC (39). The effect of K70E on resistance or enzymatic activity influenced by the fingers insertion remains to be elucidated. These results suggest that there is no correlation between the N348I and the

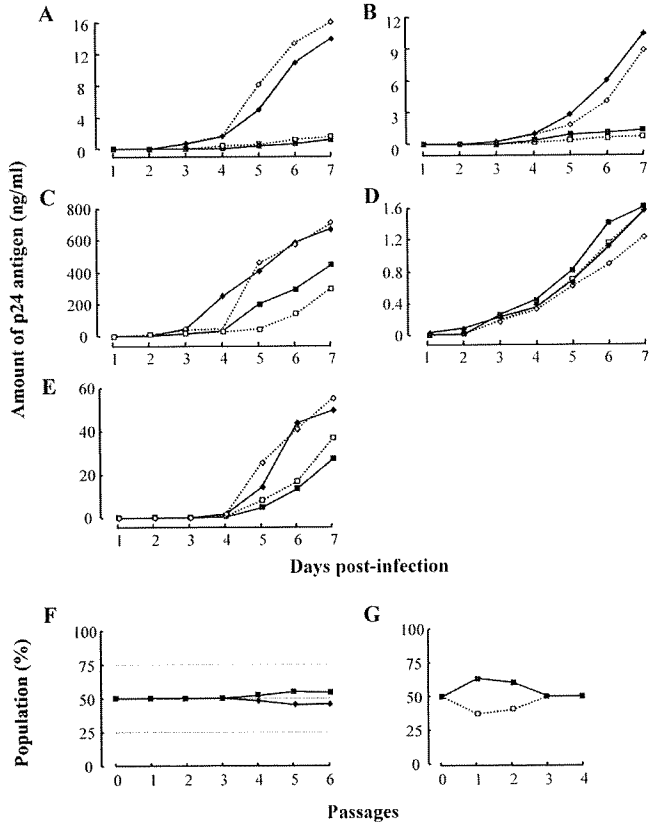

FIG. 2. Viral replication kinetics. Production of p24 antigen in culture supernatant was determined with a commercially available p24 antigen kit. Profiles of replication kinetics (p24 production) of HIV-1_{WT} (closed diamonds), HIV-1_{N348I} (closed squares), HIV-1_{I393L} (open diamonds) and HIV-$1_{N348I/I393L}$ (open squares) were determined with MT-2 (A), SupT1 (B), PM1 (C) and H9 cells (D) and PHA-stimulated PBMCs (E). Representative results from at least two (or three) independent single determinations of p24 production with newly titrated viruses are shown. A competitive HIV-1 replication assay was performed in H9 cells to compare the replication kinetics of HIV-1_{WT} (closed diamond) and HIV-1_{N348I} (closed squares) (F) and of HIV-1 N348I (closed squares) and HIV-$1_{N348I/I393L}$ (open square) (G).

finger insertion mutations. Because our studies show that N348I does not confer d4T resistance, we speculate that the fingers insertion mutation was introduced to overcome the drug pressure by d4T.

TABLE 3. Sequences of HIV-1 RT-coding region of clinical samples

No. of clones[a]	Resistance-associated and unique mutation at the indicated position									
	M41	D67	T69	K70	V75	V118	L210	T215	N348	I393
5	L	N			M	I	W	Y		L
3	L	T	SSG	E	M		W	Y	I	L
3	L	T	SSG	E	M	I	W	Y	I	L
2	L	T	SSG	E			W	Y		L
1	L	T	SSG	E			W	Y	I	L
1	L	T	SSG	E	M	I	W	Y		L
1	L				M					
1	L				M	I	W	Y	I	L

[a] The PCR product at time point 8 was subcloned and sequenced (n = 17).

TABLE 2. Drug susceptibilities of HIV-1 variants constructed by site-directed mutagenesis

Mutation[a]	EC_{50} (fold increase)[b]											
	NRTI							NNRTI				
	AZT	ddI	ddC	d4T	3TC	ABC	TDF	NVP	DLV	Loviride	EFV
WT	0.035 ± 0.01	2.3 ± 0.14	0.7 ± 0.13	3.6 ± 1.36	2.1 ± 0.2	3.4 ± 0.14	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	1.4 ± 0.38	0.003 ± 0.0008
N348I	0.24 ± 0.04 **(6.9)**	12 ± 1.0 **(5.2)**	0.74 ± 0.58 (1.1)	2.9 ± 0.21 (0.8)	1.7 ± 0.36 (0.8)	3.4 ± 1.11 (1)	0.02 ± 0.01 (0.7)	1.07 ± 0.06 **(27)**	0.22 ± 0.04 **(5.5)**	2.4 ± 0.35 (1.7)	0.005 ± 0.0005 (1.7)
I393L	0.06 ± 0.01 (1.7)	2 ± 1.37 (0.9)	0.42 ± 0.23 (0.6)	1.8 ± 1.21 (0.5)	1.5 ± 0.74 (0.7)	2.4 ± 0.95 (0.7)	0.02 ± 0.01 (0.7)	0.05 ± 0.01 (1.3)	0.04 ± 0.01 (1.0)	2.2 ± 0.4 (1.6)	0.003 ± 0.001 (1)
N348I/I393L	0.23 ± 0.03 **(6.6)**	11.3 ± 1.53 **(4.9)**	0.49 ± 0.01 (0.7)	4.2 ± 1.12 (1.2)	1.7 ± 0.40 (0.8)	2.7 ± 0.26 (0.8)	0.02 ± 0.01 (0.7)	1.02 ± 0.51 **(26)**	0.28 ± 0.06 (7)	2.6 ± 0.42 (1.8)	0.005 ± 0.001 (1.7)
M41L/T215Y	0.28 ± 0.06 **(8)**	4.5 ± 1.55 (2)	ND	ND	1.3 ± 0.25 (0.6)	ND	ND	0.05 ± 0.01 (1.3)	0.04 ± 0.02 (1)	ND	0.002 ± 0.0004 (0.7)
M41L/T215Y/ N348I	1.37 ± 0.21 **(39)**	9.9 ± 0.99 **(4.3)**	ND	ND	1.4 ± 0.20 (0.7)	ND	ND	1.11 ± 0.69 **(28)**	0.15 ± 0.06 **(3.8)**	ND	0.002 ± 0.0004 (0.7)

[a] See Materials and Methods for the construction of clones.

[b] Data are means ± standard deviations from at least three independent experiments. The relative increase in the EC_{50} value compared with that in HIV-1_{WT} is given in parentheses. Boldface indicates an increase greater than threefold. ND, not determined.

TABLE 4. Frequency of N348I acquisition in clinical isolates

Treatment group	No. of isolates (%)		*P* value[a]
	Total in group	With N348I	
AZT and/or ddI	48	6 (12.5)	<0.0001
AZT	22	2 (9.1)	0.011
ddI	16	2 (12.5)	0.006
AZT/ddI	10	2 (20)	0.002
Control	183	0	
Antiretrovirals with neither AZT nor ddI	55	0	
No antiretrovirals	128	0	
Deposited in Los Alamos database	328	3 (0.9)	0.0002

[a] The *P* value was determined by the Fisher's exact test. For the AZT and/or ddI treatment groups, values were compared with the control group. The P value for isolates deposited in the Los Alamos database was determined based on a comparison with the AZT and/or ddI treatment group.

Prevalence of N348I. We obtained viral specimens from 231 infected patients who visited our clinical center from May 1997 to July 2003 and analyzed HIV-1 sequences by direct sequencing (Table 4). The viral specimens were classified in two groups: (i) those from patients treated with AZT and/or ddI (n = 48) and (ii) those from patients treated by regimens with neither AZT nor ddI (control group, n = 183). The group treated with AZT and/or ddI was further divided into three subgroups based on the treatment received: with AZT, with ddI, and with the AZT/ddI combination (Table 4). During chemotherapy containing AZT (n = 22), ddI (n = 16), or the combination of AZT and ddI (n = 10), two patients each harbored HIV-1 with the N348I mutation. Acquisitions of N348I in all of the subgroups was statistically significant (P = 0.011, 0.006, and 0.002, respectively). In contrast, none of the patients in the control group (n = 183) harbored N348I variants. Only three variants with N348I are deposited in the Los Alamos HIV sequence database that includes subtypes B, D, and CRF14 (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html). Thus, prevalence of N348I was statistically significant in the group treated that received chemotherapy containing AZT and/or ddI ($P < 0.0001$).

Because at present the numbers of NVP- or DLV-containing regimens without AZT and/or ddI are limited in our cohort (n = 6 or n = 0, respectively), we were not able to detect acquisition of N348I in these groups. Acquisition of N348I was observed in two patients treated with EFV (Table 5). Notably, these two patients were simultaneously treated with AZT and ddI, suggesting that the significance of EFV treatment for the emergence of N348I remains unknown.

Profiles of patients infected with HIV-1 containing the N348I mutation. We further analyzed the profiles of HIV-1

TABLE 5. Profiles of patients infected with HIV-1 containing the N348I mutation

Patient	Subtype of RT region[a]	Antiretroviral treatment	Duration (mo)	HIV RNA (copies/ml)	N348I	RT mutation(s) by region	
						Polymerase subdomain	Connection subdomain[d]
Case 1[b]	D	d4T, ddI, IDV	6	6.1×10^4	+/−	M41L, D67N, V75M, L210W, T215Y	G335D, A360T
		d4T, ddI, IDV	7	ND[c]	+	M41L, D67N, V75M, L210W, T215Y	G335D, A360T
Case 2	B	AZT, ddC, NFV	1	7.9×10^3	−		A360T
		AZT, ddC, NFV	4	9×10^3	−		A360T
		AZT, ddC, NFV	6	1.2×10^4	+/−	T215N/S/Y	A360T
		AZT, ddC, NFV	10	3.5×10^4	+	D67N, K70R, T215Y[e]	A360T
		d4T, 3TC, RTV, SQV	8	<50	ND	ND	ND
Case 3	B	AZT, 3TC, RTV, SQV	7	3.5×10^5	−		A360T, A376T
		AZT, 3TC, RTV, SQV	8	1.9×10^5	+	M41L, D67N, T69D, M184V, L210L/W, T215Y	A360T, A376T
		None (interruption)	7	1.2×10^5	+	M41L, D67N, T69D, M184M/V, L210L/W, T215Y	A360T, A376T
		ABC, EFV, RTV	3	60	ND	ND	ND
Case 4	B	AZT, 3TC, ddI, EFV	3	1.7×10^3	+	M184V	
Case 5	B	d4T, ddI, RTV, SQV	23	9.9×10^3	+	M41L, L210W, T215Y	
		AZT, ddI, RTV, SQV	3	6.3×10^4	+	M41L, T69D, L210W, T215Y, K219R	
		None (interruption)	7	1.8×10^5	−		
		ABC, TDF, LPV, EFV	7	<50	ND	ND	ND
Case 6	B	AZT, ddI, EFV	3	180	−		ND
		AZT, ddI, EFV	5	540	+/−	T215T/Y	ND
		AZT, ddI, EFV	6	1.1×10^4	+	T215Y	
		None (interruption)	2	2.4×10^5	−		
		d4T, 3TC, LPV	8	<50	ND	ND	ND

[a] The RT regions were sequenced and subjected to subtype analysis (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).
[b] This patient is described in this study.
[c] ND, not detectable.
[d] G335D is an observed polymorphism in subtype D. A360I/V and A376S were reported to be AZT-resistant mutations (24).
[e] Phenotype assays were performed at 10 months for a regimen combining ddC, AZT, and NFV; resistance to AZT, ddI, and NVP was induced 52-, 6.8-, and 8.3-fold, respectively.

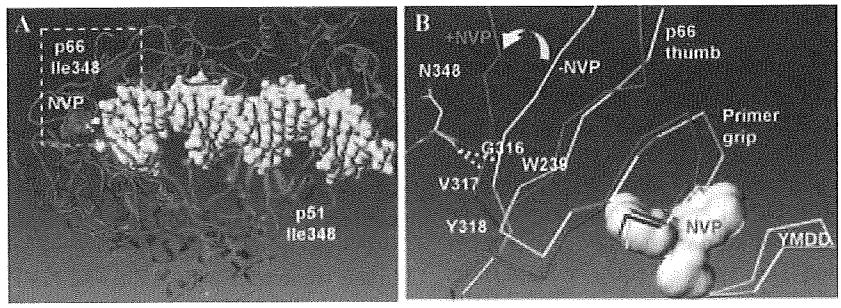

FIG. 3. Location of N348I in the modeled HIV-1 RT with NVP. (A) The N348I mutation (blue Van Der Waals volume) is shown in the connection subdomains of both p66 (purple) and p51 (cyan) subunits. The 348 residue of the p51 subunit is distant from the nucleic acid, shown as yellow Van Der Waals surfaces. In the p66 subunit (purple) the 348 residue is in a position to affect the flexibility of the p66 thumb, which in turn might affect binding of the nucleic acid. NVP is shown bound at the NNRTI binding pocket (red Van Der Waals volume). Magnification of the frame area of the enzyme is shown in panel B. (B) The main chain C=O of N348 is shown to interact with the N-H of 317 (yellow broken line) through a hydrogen bond interaction. Binding of NVP (white ball) repositions the p66 thumb subdomain with respect to (i) the polymerase active site (β6-β9-β10) that contains the three catalytic aspartates and the YMDD motif and (ii) the primer grip (β12-β13) of p66. The movement of the thumb subdomain is in a hinge-like motion that is based at the position where residue 348 interacts with residue 317.

with N348I from the six infected patients described in Table 4. The results of this analysis are shown in Table 5. The RT regions were sequenced and subjected to analysis with the software Genotyping, which uses the BLAST algorithm to determine homologies with known subtypes (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). HIV-1 variants in case 1 belonged to subtype D, and the others belonged to subtype B. All six patients received therapy containing AZT and/or ddI. Among them, two patients (cases 4 and 6) were under therapy with EFV. However, none of them was treated with NVP or DLV. The five N348I-containing variants were in the presence of TAMs that emerged during the therapies. TAMs in case 1 and some TAMs (M41L, L210W, and T215Y) in case 5 seemed to be induced by d4T, not by AZT. In case 3, the 3TC resistance mutation M184V that attenuates TAM-induced AZT resistance (24) was present together with N348I. Similarly, in case 4, M184V may confer AZT hypersusceptibility. In case 6, N348I was present together with a classical AZT resistance mutation, T215Y. Thus, except for case 5, even under AZT-containing therapy, the HIV-1 resistance level to AZT and ddI seemed to be intermediate and weak, respectively. Additionally, viral load in cases 2, 3, 5, and 6 dramatically decreased after introduction of a new regimen without AZT and/or ddI. These results indicated that N348I may enhance AZT resistance and at least act as a primary mutation for ddI.

In these six patients, HIV-1 with the G335D mutation was observed only in case 1. In the Los Alamos HIV sequence database, G335D has been observed in 77% of subtype D HIV-1 isolates (n = 35). A360T was detected in two isolates of subtype B and one isolate of subtype D and was observed in 13 and 51% of drug-naïve isolates of subtypes B and D, respectively. This suggests that A360T is also one of the polymorphisms. The A360V or A360I mutation has been reported to have a modest effect on AZT resistance (28). Meanwhile, none of N348I-containing subtype B variants (n = 5) had mutations associated with AZT resistance in the connection subdomain (28) (Table 5).

Molecular modeling. Residue 348 is located close to the hinge site of the thumb subdomain. Mutations at the virus level affect both subunits of RT. Figure 3 shows that residue 348 of the p51 subunit is located remotely from the polymerase active site (~60 Å) and from the NNRTI binding pocket (~55 Å). Furthermore, it is not in close proximity to the interface of the two subunits (~20 Å) or the DNA in the nucleic acid binding cleft (~15 Å). On the other hand, residue 348 of the p66 subunit is proximal to the NNRTI-binding site and the nucleic acid binding cleft. These relative distances suggest that it is more likely that the interactions involve mainly residue 348 of the p66 subunit. Subunit-specific biochemical analysis would determine the precise contribution of the N348I mutation in each subunit to the drug resistance phenotype. In the p66 subunit, the main chain of the 348 residue interacts through a hydrogen bond with the main chain of V317 of the p66 thumb subdomain (Fig. 3). To determine the degree of flexibility of this part of the structure of RT, we superposed 23 structures of RT complexes. The comparison revealed measurable differences. The length of the amide bond between the main chain C=O of residue 348 and N-H of V317 varies considerably (from 2.5 to 3.6 Å), suggesting a flexibility at the junction of the connection, thumb, and palm subdomains. It is likely that the N348I mutation affects the interactions of this residue with a number of neighboring residues. In the RT/DNA/deoxynucleoside triphosphate or RT/DNA/TDF structures of ternary catalytic complexes (PDB code 1RTD or 1T05, respectively), the change of N348 to a more hydrophobic Ile would improve the hydrophobic interactions with T351 of the p66 connection subdomain and with G316 and I270 of the p66 thumb subdomain. In other structures of complexes of RT with various NNRTIs (PDB codes 1S1X, 1S6P, 1S1U, 1S1T, 1S1W, 1TKZ, 1TKX, 1TL1, 1SUQ, 1SV5, 1HNI, 1HQU, and 1HNV), residue W239 appears to be in the vicinity of these residues and likely to be affected directly or indirectly by the N348I mutation. Notably, residue W239 interacts through P-P interactions with Y318, which has been involved in resistance to NNRTIs (NVP and DLV) (19, 33).

DISCUSSION

Two previous reports have shown that two rare mutations, Q145M/L and Y181I, can confer cross-resistance to some NRTIs and NNRTIs (31, 32). N348I appears to be the first reported high-prevalence amino acid mutation to confer resistance to multiple members of the NRTI and NNRTI families. N348 is highly conserved in HIV-1 strains, including subtype O. Interestingly, the equivalent residue in HIV-2 and other retroviruses is an isoleucine (Los Alamos Sequence Data Base, http://hiv-web.lanl.gov/content/hiv-db/). Similarly, WT HIV-2 RT resembles NNRTI-resistant HIV-1 RTs at the NNRTI binding pocket region, e.g., V/I at 181 and L at 188 (34). Any of these differences from the HIV-1 enzyme, including N348I, may contribute to the observed NNRTI resistance of the HIV-2 RT. The significance and role of I348 in the natural resistance of HIV-2 to NNRTIs and susceptibility to NRTIs remain to be elucidated by further experiments.

Recently, Shafer et al. proposed criteria for evaluating the relevance of mutations to drug resistance based on extensive resistance surveillance data (37). In this review the mutations related to drug resistance were assessed by the following: (i) correlations between a mutation and treatment (whether the drug therapy selects for the mutation), (ii) correlations between a mutation and decreased in vitro drug susceptibility, and (iii) correlations between a mutation and a diminished in vivo virologic response to a new antiretroviral regimen.

Regarding the first criterion, we showed that the N348I mutation was induced by AZT and/or ddI treatment (Table 4). For the second criterion, we showed that N348I decreases susceptibility to AZT, ddI, NVP, and DLV (Table 2). The AZT and ddI resistance of the N348I clone was comparable to that of M41L/T215Y and L74V, respectively. Additionally, N348I showed 27-fold increased resistance to NVP. Regarding the third criterion, our data on patient viral load levels shown in Table 5 indicate that N348I affected the clinical outcome. Specifically, in case 6, the viral load clearly increased upon acquisition of N348I. Moreover, dramatic decreases in viral load were observed after introduction of a new regimen without AZT and/or ddI, especially in cases 2, 3, 5, and 6. Hence, the N348I mutation meets the accepted criteria for being a drug resistance mutation.

At present, it is not possible to accurately compare the incidence of N348I with that of other resistance mutations. Genotypic analysis of the largest and most recent drug resistance surveillance examined 6,247 patients treated with well-characterized RTIs, mainly performed within amino acids 1 to 240 of the RT region (35). In this surveillance, the incidences of the Q151M complex and fingers insertion were 2.6 and 0.5%, respectively. Because the connection subdomain is located outside the region sequenced in the majority of genotypic assays, only limited data are available for connection subdomain mutations such as G333E/D and N348I. Nonetheless, the incidence of N348I in our cohort is higher than other MDR mutations such as that of the Q151M complex and the insertion mutations. Furthermore, prevalence of N348I in a Canadian cohort (11.3%) (42) is comparable to that in our Japanese cohort.

In the patient case presented in Fig. 1, there is strong evidence that N348I was not present during and at least 6 months after cessation of NNRTI-based therapy. Still, because of the limited number of such cases in our cohort, it remains unclear if N348I can be induced by NNRTI-containing regimens. According to the Stanford HIV drug resistance database, the incidence of N348I in patients treated with NNRTIs is 5.8% (n = 13/224), significantly higher than in the untreated group (0.1%; n = 2/1095, $P < 0.0001$). We report here that N348I confers significant and moderate resistance to NVP and DLV, respectively. Most recently, Yap et al. also reported that combined treatment with AZT and NVP was associated with increased risk in the emergence of N348I (42). They mention that other mutations, e.g., K103N, may further enhance N348I-induced resistance to EFV. Thus, it is possible that HIV-1 also acquires N348I under NNRTI-containing therapy. Further experiments and surveillance are needed in patients treated with NNRTI(s) as well as NRTIs.

Mutations at multiple residues are present in the MDR variants of the Q151M and the fingers insertion complexes. Q151M complexes typically contain at least four mutations, including V75I, F77L, and F116Y in addition to Q151M (21). Insertion complexes generally contain an insertion of six bases that code for two amino acids in the background of the classical AZT resistance backbone such as T215Y (41). These results suggest that genetic barriers to developing these MDR mutations appear to be high, consistent with their low incidence (35). Genetic barriers to the G333D/E complex also seem to be high, since G333D/E requires other TAMs to develop this certain resistance phenotype (7). In contrast, a single nucleotide substitution (AAT to ATT) is sufficient to develop the N348I mutation, indicating that the genetic barrier to N348I is low. This may contribute to an increased prevalence of N348I during prolonged chemotherapy with AZT and/or ddI.

The disappearance of N348I was relatively rapid following interruption of treatment (Fig. 1 and Table 5). This was consistent with the observed replication kinetics of N348I HIV-1 where strong impairment was observed in MT-2 and SupT1 cells (Fig. 2). However, in PM1 cells and PHA-stimulated PBMCs, this reduction was moderate, and in H9 cells little reduction was observed. Since both PM1 and H9 cells were originally derived from the same T-cell line, Hut78 (25, 26), some properties for HIV replication may be identical. Availability of deoxynucleoside triphosphates or some cellular factors may compensate the effect of N348I on RT activity, suggesting that some cell populations in patients might harbor HIV-1 with N348I due to its comparable replication kinetics with the WT.

How might the N348I mutation affect resistance to NRTI and NNRTI inhibitors that act with entirely different mechanisms and target different binding sites? Theoretically, it is possible that the N348I mutation at either p66 or p51 or both subunits is responsible for the resistance phenotype. It is also possible that NRTI and NNRTI resistance do not involve the same subunit. However, the N348I mutation in p51 is 50 to 60 Å away from the polymerase active site and the NNRTI binding pocket where the affected inhibitors are expected to bind. Similarly, the mutation site in p51 is 15 to 20 Å away from the interface of the two subunits or the DNA binding cleft. Meanwhile, the mutation site in the p66 subunit is close to the NNRTI-binding pocket and the nucleic acid binding cleft. Hence, it is more likely that the effects of the N348I mutation

are mediated through the p66 subunit mutation, although an involvement of the mutation at the p51 subunit currently cannot be ruled out and should be addressed by biochemical experiments.

In terms of NNRTI resistance, our molecular modeling analysis is consistent with a hypothesis that the mutation is likely to affect the flexibility and mobility of the p66 thumb subdomain. Extensive crystallographic work with HIV-1 RT in several forms, including an unliganded form, in complex with DNA substrates or NNRTIs has revealed that during the course of DNA polymerization, the p66 thumb subdomain undergoes major conformational motions that are critical for efficient catalysis. Alignment of multiple structures of HIV RT suggests that the p66 thumb moves as a rigid body with its base hinged to the palm subdomain exactly near residue 348 (Fig. 3). Residue 348 is proximal to, and likely to affect, the relative interactions between residues of the p66 connection (T351) and p66 thumb subdomains (V317, I270, P272, W239, and eventually Y318). The proximity of residue 348 to this hinge region leads us to believe that changes imparted by the N348I mutation alter the mobility and flexibility of the thumb subdomain. Subtle changes in the interactions between V317 and N348 may also reposition W239 and its neighboring Y318 in the NNRTI-binding pocket. Interestingly, the Y318F mutation affects NNRTI resistance in a similar way as N348I: it decreases susceptibility to NVP and DLV but not to EFV (19, 33). Biochemical binding experiments of RTs with NNRTIs would directly evaluate this hypothesis.

The effect of the N348I mutation on NRTI resistance cannot be rationalized by direct interactions of the mutated residue with the NRTI binding site. It is tempting to speculate that minor changes in the p66 thumb subdomain hinge motions also have minor effects on the positioning of the nucleic acid, which in turn affects the ability to discriminate between NRTI and the normal substrate by an as yet undefined mechanism. However, direct biochemical experimental evidence will be needed to determine the precise molecular details of the specific mechanisms of NRTI resistance.

It has been proposed previously that an imbalance between reverse transcription and RNA degradation plays an important role in NRTI resistance (25). Pathak and colleagues proposed that connection subdomain mutations may result in a slower RNase H reaction, and this in turn may provide an increased time period available for AZT excision, especially with TAMs (28–30). In the case of N348I, Yap et al. recently reported that N348I decreases RNase H enzymatic activity (42). At present, available evidence is consistent with a model in which these connection subdomain mutations alter the affinity of the RT for template/primer, enhance nucleoside excision, and reduce template switching.

Several studies, including recent work by Delviks-Frankenberry et al. and Brehm et al. (4, 11), highlighted the necessity to expand sequencing analysis to include the connection and RNase H subdomains. This contention is further supported by results in this work and by others (16, 28, 42, 43) showing that mutations at the connection subdomain influence susceptibility to some antiretroviral drugs. Hence, there is a growing interest in obtaining genotypic information from expanded areas of RT that would be useful for a more complete analysis of HIV drug resistance. Interestingly, already two out of four commercially available genotypic and phenotypic assay kits are designed to include in their analysis at least part of the connection subdomain (Antivirogram by Virco up to RT residue 400 and ViroSeq by Abbott/Celera Diagnostics up to RT residue 335).

The present study identifies N348I as a MDR mutation in HIV-1 RT. This knowledge provides information that may be useful in designing more efficient therapeutic strategies that can improve clinical outcome and help prevent the emergence of MDR variants, especially in salvage therapy. This work further highlights the functional role of the HIV-1 RT connection subdomain in drug resistance. Future studies that focus on the structural and biochemical properties of connection subdomain RT mutants should reveal the molecular details of NRTI and NNRTI drug resistance caused by connection subdomain residues.

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Original article

HLA-A*2402-restricted HIV-1-specific cytotoxic T lymphocytes and escape mutation after ART with structured treatment interruptions

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Abstract

Although a limited duration of immune activation of structured treatment interruptions (STIs) has been reported, the immune escape mechanism during STIs remains obscure. We therefore investigated the role of three immunodominant cytotoxic T lymphocyte (epitopes) in 12 HLA-A*2402-positive patients participating longitudinally during the clinical study of early antiretroviral treatment (ART) with five series of structured treatment interruptions (STIs). The frequency of HLA-A*2402-restricted CTLs varied widely and a sustained CTL response was rarely noted. However, a Y-to-F substitution at the second position in an immunodominant CTL epitope Nef138-10 (Nef138-2F), which was previously demonstrated as escape mutation, was frequently detected in seven patients primarily and emerged in the remaining five patients thereafter, and the existence of escape mutations was correlated with high pVL levels early in the clinical course. These findings suggest that escape mutation in the immunodominant CTL epitope may be one of the mechanisms to limit HIV-1-specific immune control in STIs.

Keywords: Structured treatment interruptions; Cytotoxic T lymphocyte; HLA-A*2402; Escape variant

1. Introduction

Structured treatment interruption (STI) is considered one of the immune stimulatory interventions for HIV-1 infection, based on the hypothesis that viral rebound during treatment interruption might induce HIV-specific immune responses [1–3]. Since the 1999 case report of the early-treated patient who achieved sustained viral suppression without highly antiretroviral therapy (HAART) after two occasional treatment interruptions [1], the STI strategy has been studied in various clinical settings [4–7]. Because cytotoxic T lymphocytes (CTLs) play a critical role in the control of HIV-1 replication and HIV-specific CD4+ T-cell response is important to maintain effective HIV-1-specific CTLs [8-11], early treatment that can preserve HIV-1-specific-CD4+ T cells is considered to have the greater impact on STI in early infection than in chronic infection [11–13]. However, the majority of previous STI trials revealed the limitation of immune activation with risk of viral resistance [4,14,15] and the mechanisms of viral control failure in STI strategy have remained unclear.

Viral mutation in immunodominant epitopes is one of the obstacles to HIV-1 vaccine development [16–21]. Since HIV-1-specific T-cell responses are restricted by HLA alleles, its escape variant can be transmitted and adopted in populations sharing some dominant HLA alleles [19–21]. In Japan where HLA-A*2402 is the most frequent HLA class I allele with 70% prevalence, HLA-A*2402-restricted CTLs and its immunodominant epitopes have been extensively assessed [22]. Nef138-10, which has been proved previously as an HLA-A*2402-restricted CTL epitope provoking strong cytolytic activity [22], is one of the immunodominant CTL epitopes in HLA-A*2402-positive Japanese patients [21,22].

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Our previous study showed that a Y-to-F substitution at the second position in Nef138-10 epitope (Nef138-2F) impairs the ability of the Nef138-10-specific CTLs to suppress HIV-1 replication, indicating that Nef138-2F is an escape mutation from CTLs [23]. Since Nef138-2F is observed in both HLA-A*2402-positive and -negative patients, Nef138-2F variant may be stable and adopted at a population level [21].

In the present study of early antiretroviral treatment with five series of STIs for HLA-A *2402 positive Japanese patients, we investigated the longitudinal magnitudes of HIV-1-specific HLA-A*2402-restricted CTLs by using HLA-epitope tetramer binding assay and sequenced the most immunodominant epitopes Nef138-10 to evaluate whether escape mutation might negatively influence viral control in an STI study.

2. Methods

2.1. Study design and patient population

This trial was designed as a prospective study at the AIDS Clinical Center, International Medical Center of Japan. Between November 2000 and December 2001, patients with early HIV infection, with or without acute retroviral symptoms, were recruited. Early HIV infection was confirmed within 6 months before recruitment by a documented history of seroconversion in enzyme-linked immunosorbent assay (ELISA) or longitudinal increase of bands in Western blot test. Patients with active opportunistic infections or psychological disorders, or those treated with immunomodulatory agents were excluded. Antiretroviral therapy was initiated after obtaining a signed informed consent. The first-choice regimen for this study consisted of stavudine, lamivudine and indinavir boosted with ritonavir, but the patient was allowed to use other antiretroviral drugs when the first regimen could not be tolerated. To avoid emergence of drug resistance to indinavir, ritonavir-boosting was stopped more than 1 week before treatment interruption. The duration of treatment interruption was fixed for 3 weeks. The first treatment was interrupted after more than 3 months of HAART, when CD4+ cell count was $>500/mm^3$ and plasma viral load (pVL) had been <50 copies/ml for at least 1 month. Other interruptions were also carried out when pVL became <50 copies/ml and CD4+ cell count was $>300/mm^3$. Five series of STIs were scheduled during the treatment.

The study protocol was approved by the institutional ethical review boards (IMCJ-H13-10).

2.2. Monitoring and sample collection

Patients were monitored monthly during HAART and at approximately a 4-month interval after treatment discontinuation. Unscheduled visits were permitted according to clinical needs. At each visit, clinical assessment and routine laboratory tests were performed. Blood specimens were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes, separated into peripheral blood mononuclear cells (PBMCs) and plasma, and stored at −80 °C for assessment of HIV-1-specific CTLs and sequence of the dominant epitope region. pVL was quantified by using the Amplicor HIV-1 Monitor test 1.5 (Roche Diagnostics, Indianapolis, IN) with a detection limit of 50 copies/ml. Antiretroviral drug resistance-associated mutations were examined at baseline and after HAART including STIs in all 26 participants. Each mutation was identified according to the revised August 2006 International AIDS Society Resistance-USA Panel [24].

2.3. HLA typing and epitope-HLA-A*2402 tetramer binding assays

High-resolution HLA class I typing was performed by a PCR-sequence-specific primer method. If HLA-A*2404 was positive, HIV-1 specific CTLs were investigated by using peptide-HLA-A*2402 tetrameric complex synthesized as described previously [21,22,25]. Purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C-terminus of the heavy chain, and then mixed with phycoerythrin (PE)-conjugated avidin (extravidin-PE; Sigma–Aldrich, St. Louis, MO) at a molar ratio of 4:1. Cryopreserved PBMCs ($0.5-1 \times 10^6$ cells) were stained by the tetramer at 37 °C for 30 min. After double washing with washing buffer (10% fetal calf serum in RPMI 1640), the cells were stained by fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 mAb (BD Biosciences, San Jose, CA) at 4 °C for 30 min. The cells were then washed twice and analyzed using a FACS Calibur with Cell Quest software (Becton Dickinson, San Jose, CA). Based on our previous study [22], three immunodominant epitopes of HLA-A*2402 restricted CTLs; Nef138-10, Gag28-9 and Env584-9, were chosen for this assay. Since we found a high frequency of Y-to-F substitution at the second position in Nef138-10 gene (Nef138-2F) which has been suspected as an escape variant in previous studies [21], Nef138-2F-specific CTLs (Nef138-2F-CTLs) were also measured by tetramers using Nef138-2F variant alone and by competitive double staining using two types of tetramers of both wild type and Nef138-2F variant to compare the frequencies of the two types of HIV-1-specific CTLs.

2.4. Sequence analyses of Nef138-10 gene

For evaluation of escape variants from CTLs, we sequenced the region coding Nef138-10, which is the immunodominant HLA-A*2402-restricted epitope, while Nef138-2F has been suspected as escape mutation in this epitope, using the method described here. Total RNA was extracted from plasma with a High Pure viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by RT–PCR with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan) to amplify the HIV-1 Nef DNA segment (2341 bp) as described previously [21]. The PCR products were purified with SUPREC-02 (TaKaRa Shuzo) 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 5.1 (Software Development, Tokyo).

2.5. Statistical analysis

Data from patients who completed the treatment protocol including five series of STIs were analyzed. Before analysis, pVL data were log-transformed and undetectable pVL (<50 copies/ml) was considered equivalent to 50 copies/ml. The Mann–Whitney *U*-test was used to compare the pVLs determined every 3 months after treatment cessation to the pVLs of 279 untreated chronic HIV-1 patients in order to assess the durability of viral suppression. The correlation between pVL and percentage of CTLs was assessed by simple regression analysis. Statistical analyses were performed using SPSSII software package for Windows, version 11.0J.

3. Results

3.1. Characteristics of participants

During the enrollment period, 432 new patients were referred to our clinic. Of these, 32 met the criteria of early HIV-1 infection and 6 were excluded due to psychological problems or taking systemic steroid therapy for symptoms associated with acute retroviral syndrome. All 26 recruits were Japanese infected with HIV-1 by sexual intercourse, and 24 were men (92%). The mean age of patients was 35.0 years (range, 21–56 years). The mean pVL at baseline was 5.21 $\log_{10}$ copies/ml (range, 3.28–6.91 $\log_{10}$ copies/ml) and the mean CD4+ cell count at baseline was 413/mm^3 (range, 49–1156/mm^3). Twenty-five patients presented with wide-range clinical symptoms of acute retroviral syndrome. Fifteen out of 26 participants completed the treatment protocol including five series of STI. HAART had to be continued in four patients because CD4+ cell counts had never stabilized above 300/mm^3 despite more than 6 months of treatment. The other seven patients discontinued the treatment protocol after less than five STIs due to adverse events, adherence problems, or no specific problems.

In the protocol-completed 15 patients, 14 were men (92%). The mean age was 34.0 years (range, 21–56 years). At baseline, the median pVL was 5.14 $\log_{10}$ copies/ml (range, 3.28–6.91 $\log_{10}$ copies/ml) and the median CD4+ cell count was 475/mm^3 (range, 245–990/mm^3). The demographic, immunological, and virological factors before initiation of HAART of the protocol-completed group were not statistically different from those of the uncompleted group (Mann–Whitney *U*-test) (data not shown), although baseline CD4+ cell counts of four ART-continued patients: 49, 185, 210, and 351/mm^3 respectively seemed lower than those who completed the treatment protocol. Twelve (80%) patients were positive for HLA-A*2402 and its incidence was similar to those reported previously in Japanese population [21,22]. No specific HLA genotypes that are known to influence the clinical course of HIV infection such as HLA-B*27, HLA-B*57 and HLA-B*35 (except B*3501) [26] were detected in participants. The median length of follow-up after treatment cessation was 961 days (range, 462–1255 days).

No resistance-associated mutations were identified among all the 26 participants at study enrollment except one who had M184V, D30N and L90M mutations despite good virologic responses throughout HAART. There was no increase in resistance-associated mutations during and after five STIs in all participants (data not shown).

3.2. Plasma viral load and CD4+ cell count in protocol-completed 15 patients

Fig. 1 shows serial changes in median pVLs and CD4+ cell counts in protocol-completed 15 patients. Peaks of viral

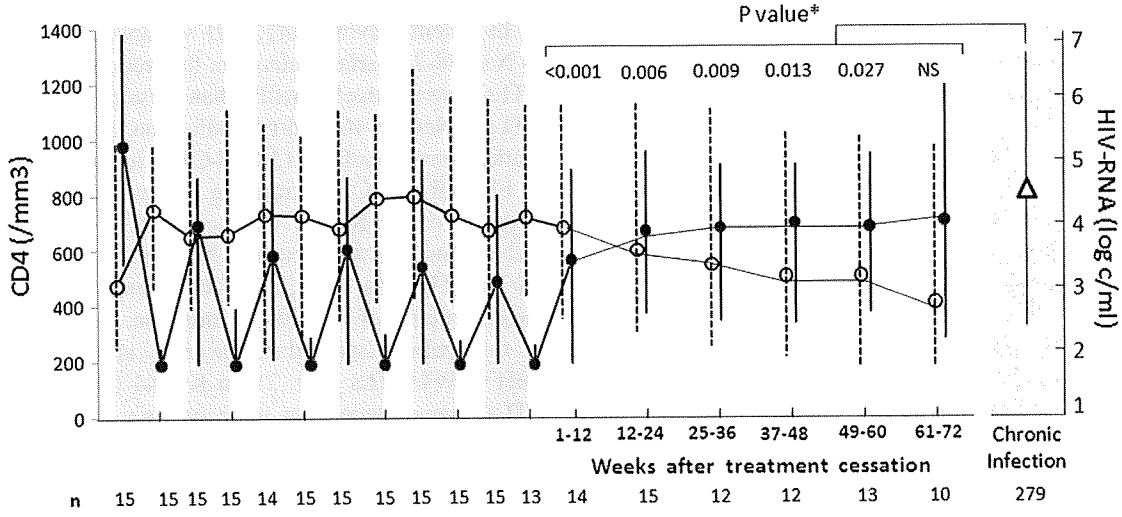

Fig. 1. Serial changes in plasma viral loads and CD4+ cell counts of 15 protocol-completed patients. Plasma viral loads (pVLs) and CD4+ cell counts are expressed as median of 15 protocol-completed patients; at baseline, at the times of treatment interruption, at the peaks of pVL rebound during structured treatment interruption and at every 12 weeks after treatment cessation. Open circles: CD4+ cell counts; solid circles: pVLs; triangle: the median pVL of 279 untreated chronic HIV-1 patients who were referred to our clinic during the study and whose CD4 count was >200/mm^3. Vertical lines provide the ranges with dotted lines in CD4+ cell counts and with light lines in pVLs. Shaded area: time on antiretroviral therapy; unshaded area: time off therapy. Numbers of patients whose data were evaluated at each time point appear at the bottom of the graph. *pVLs of every 12 weeks after treatment cessation were compared to the pVLs of 279 untreated chronic HIV-1 patients by Mann–Whitney *U*-test.

rebounds during treatment interruptions decreased gradually. The pVLs of every 12 weeks after treatment cessation were under 4 $\log_{10}$ copies/ml in most of the patients and they were significantly lower for 60 weeks than the pVLs of 279 untreated chronic HIV-1 patients in our clinic. However, pVLs gradually increased and there was no difference at week 61–72 from pVLs of chronically infected patients. The proportion of patients with a favorable viral control whose median pVL at every 12 weeks after treatment cessation were less than 4.0 $\log_{10}$ copies/ml was 66% in the first 12 weeks but the proportion decreased to 33% in the 61–72 weeks. Along with the increase in pVL, CD4+ cell counts declined after treatment cessation and one patient (KI-134) required restart of HAART because CD4+ cell count decreased below 200/mm^3 at week 52. None of the patients developed episode of opportunistic infections or HIV-related diseases throughout this study.

*3.3. Plasma viral loads and frequency of HLA-A*2402-restricted CTLs*

We investigated induction of 3 HLA-A*2402-restricted immunodominant epitope-specific CTLs in 12 patients with HLA-A*2402 by using the corresponding tetramers. Fig. 2 shows the serial changes in HLA-A*2402-restricted HIV-1-specific CTLs. Overall, the frequency of HLA-A*2402-restricted CTLs varied widely among the patients and a sustained CTL response was rarely noted. We investigated the correlation between pVLs at every 12 weeks after treatment cessation and frequency of HLA-A*2402-restricted CTLs according to the epitope. None of Nef138-10-, Gag28-9- or Env584-9-specific CTLs was statistically correlated to pVLs (Fig. 3A).

3.4. Effect of Nef138-10 escape mutation on suppression of HIV replication

A Y-to-F substitution at the second position of Nef138-10 (Nef138-2F) has been suspected as an escape mutation from HLA-A*2402-restricted Nef138-10-specific CTLs in a previous study [21]. In fact, we recently demonstrated that Nef138-10-specific CTLs fail to suppress replication of Nef138-2F mutant [23]. We therefore performed serial sequence analyses of Nef138-10 epitope and investigated whether this 2F mutation is responsible for the limited duration of viral suppression. As shown in Table 1, we found high frequency of this mutation. Seven out of 12 patients had Nef138-2F variant in viral RNA or proviral DNA in the earliest samples (KI-091, KI-126, KI-134, KI-144, KI-150, KI-154 and KI-163). The Nef138-2F variant was not detected in the earliest samples of the other five patients (KI-092, KI-099, KI-102, KI-158 and KI-161) and these patients were considered to have Nef138-10 wild-type infection except a T-to-C substitution at the fifth position (Nef138-5C) in KI-099 which has also been suspected as one of the escape variants from Nef138-10-specific CTLs in a previous study [21], and an L-to-I substitution at the forth position (Nef138-4I) in KI-161. However, Nef138-2F mutation was detected at the latter stage in all the other five patients.

We speculated that Nef138-10-specific CTLs can control replication of HIV-1 in patients who had been infected with Nef138-WT virus. Therefore we compared pVLs according to the existence of escape mutants Nef138-2F or 138-5C at the earliest sample drawn during early phase of infection before treatment initiation. As shown in Fig. 3B, the pVLs between 13 and 36 weeks were significantly lower in the other four patients who were confirmed as Nef138-WT or Nef138-4I infection than in the remaining eight patients who had Nef138-2F or Nef138-5C variant in the earliest samples, which has been suspected as an escape variant from Nef138-10-specific CTLs in a previous study. These indicate that Nef138-10-specific CTLs control replication of wild-type virus but the presence of either Nef138-2F or Nef138-5C negatively influences viral control.

3.5. Nef138-2F variant specific CTLs

We found Nef138-WT-tetramer and Nef138-2F-tetramer bound to both Nef138-WT-specific CTL clones and Nef138-2F-specific CTL clones. In addition, Nef138-WT-tetramer had stronger affinity to Nef138-WT-specific CTL clones than Nef138-2F-specific CTL clones (Fig. 4A) and vice versa (our unpublished work). Therefore, the double-staining assay using both tetramers simultaneously was performed to differentiate the two types of CTLs.

The frequencies of the two types of CTLs are shown in Table 1. In patients negative for Nef138-2F or Nef138-5C initially, Nef138-WT-CTLs were detected early after the treatment cessation (KI-092, KI-102, KI-158 and KI-161) but declined after evolution of Nef138-2F (KI-092, KI-102, and KI-161). Although only a slight elevation of Nef138-2F-CTLs was noted after emergence of Nef138-2F (KI-092 and KI-161), the magnitude was smaller than that of Nef138-WT-specific CTLs before emergence of Nef138-2F.

In patients having Nef138-2F variant initially and suspected as Nef138-2F variant infection, the frequencies of Nef138-2F-CTLs were relatively smaller than those of Nef138-WT-specific CTLs in Nef138-10 wild-type infection, except KI-144 who had marked increase of Nef138-2F-CTLs in week 37.

Fig. 4B and C illustrate the clinical courses of two representative cases; KI-161 was non-Nef138-2F variant infection and KI-144 was suspected as Nef138-2F variant infection. In KI-161 (Fig. 4B), Nef138-WT-CTL response diminished after the emergence of Nef138-2F mutation. Interestingly, the pVL of this patient seemed to increase along with the fall in Nef138-WT-CTLs (Fig. 2). In KI-144 (Fig. 4C), Nef138-2F-CTLs were induced but there was no suppression of pVLs. These results indicate that either infection or emergence of Nef138-2F variant might limit the CTL induction.

4. Discussion

In this study, we could not demonstrate the lowered set-point pVLs in patients who received HAART with five series of STIs in early HIV-1 infection. Previous studies revealed that a vigorous HIV-1-specific CD4 response is associated

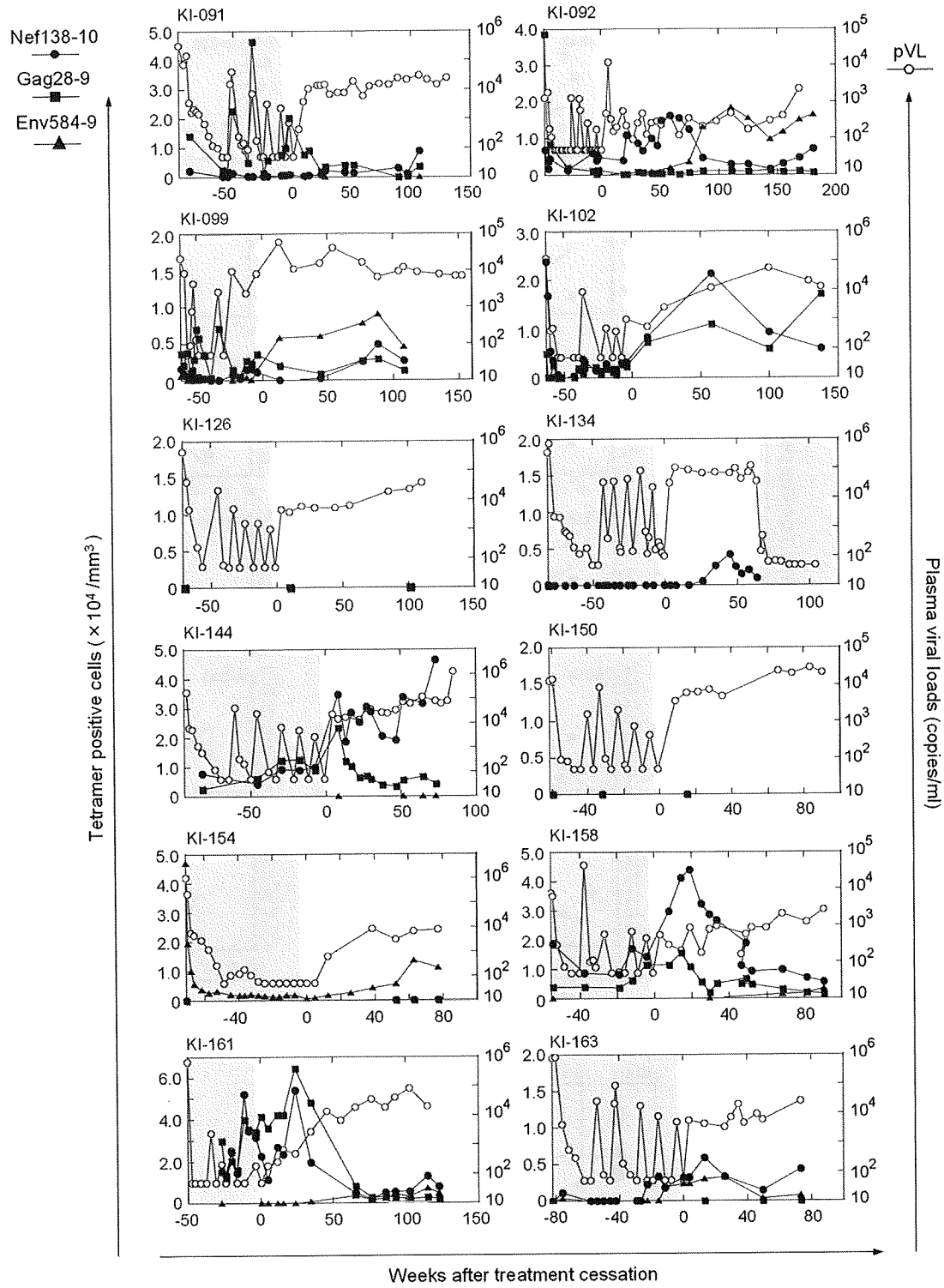

Fig. 2. Frequencies of HLA-A*2402 restricted HIV-1-specific CTLs determined by tetramer binding assay. HLA-A*2402 restricted HIV-1-specific CTLs in PBMCs were determined by using tetrameric complexes of HLA-A*2404 and each of the three types of epitopes. Solid circle: Nef138-10-specific CTL; solid squares: Gag28-9-specific CTL; solid triangles: Env584-9-specific CTL; open circles: plasma viral load. Shaded area: time on antiretroviral therapy; unshaded area: time off therapy.

with a slower disease progression [8–11]; however, despite some reports of boosted immunological responses in acutely treated patients, the evidence of clinical benefits of early treatment has not been established [12,13]. In line with these trials of early initiation of HAART with or without STI, the CTL responses in our study were mostly transient and did not correlate with pVL levels.

We adopted HLA-epitope tetramer analysis for evaluating CTL responses, which provides specific information on HLA class I allele and HLA-restricted epitopes, because CTL

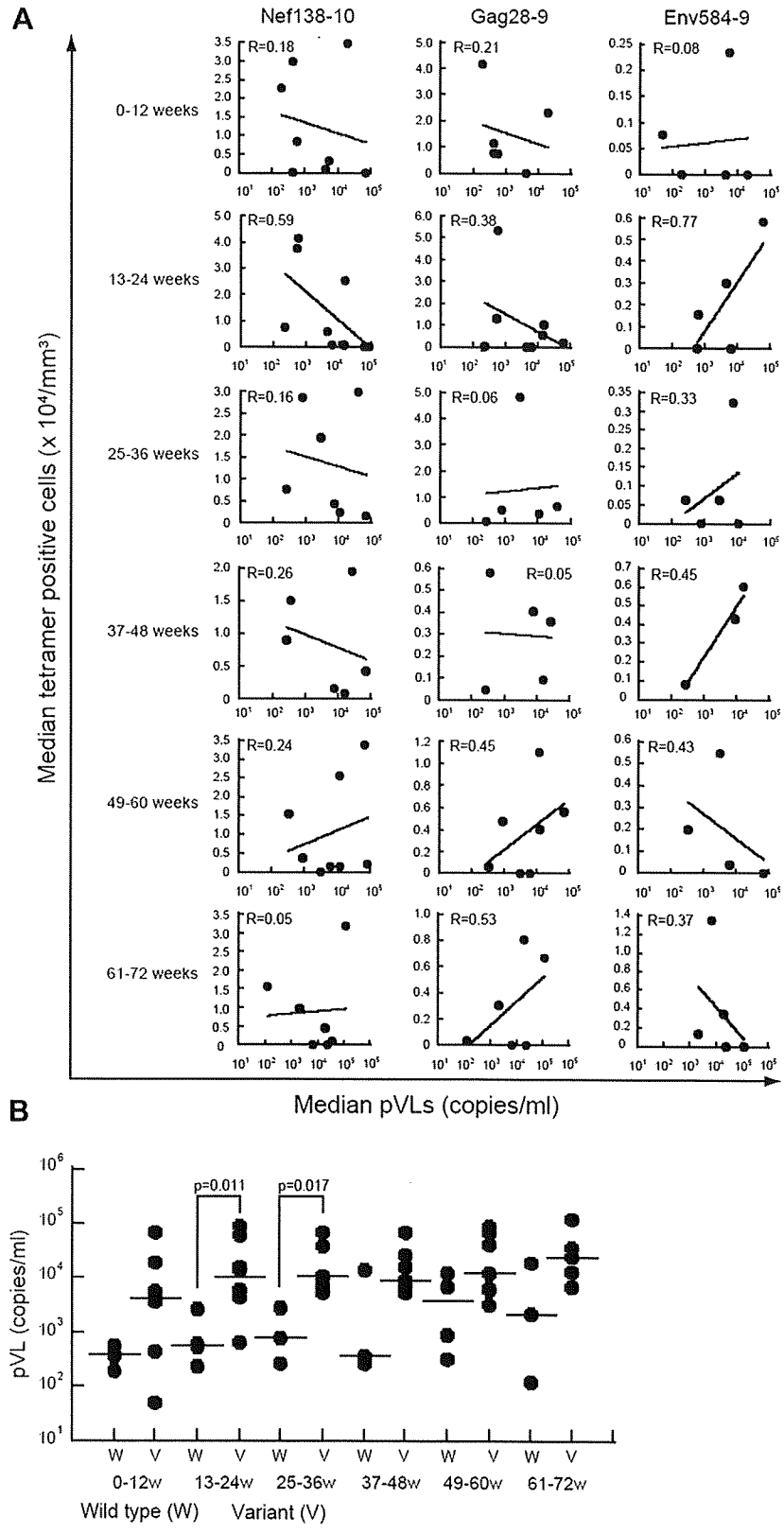

Fig. 3. (A) Plasma viral loads and frequency of HLA-A*2402-restricted HIV-1-specific CTLs. The correlation between the pVL values of every 12 weeks after treatment cessation and frequency of HLA-A*2402-restricted CTLs was assessed by simple regression analysis according to the epitope in 12 HLA*A2402-positive patients. None of Nef138-10-, Gag28-9- or Env584-9-specific CTLs was statistically correlated to pVLs at any time point. R: correlation coefficient. (B) Plasma viral loads and initial type of virus. pVL was compared according to the existence of escape variant in the earliest sample drawn during early phase of infection. Wild type group (W) includes four patients: KI-092, KI-102, KI-158 and KI-161. Variant type group (V) includes eight patients: KI-091, KI-099, KI-126, KI-134, KI-144, KI-150, KI-154 and KI-163, having Nef138-2F or Nef138-5C, which were previously reported as escape variants, in viral RNA or proviral DNA in the earliest samples. The pVLs between 12 and 36 weeks were significantly higher in Variant type group than in Wild-type group. Horizontal lines: median values.

Table 1
Nef138-10 sequence and Nef138-specific CTLs in HLA-A*2402 positive patients

Patient ID	Time (weeks)[a]	Sample	Nef138-10 sequence (RYPLTFGWCF)	Tetramer positive cell (% in CD8+ cells)	
				Wild type	2F
KI-091	−55	Proviral DNA	-F————	NA	NA
	21	RNA	-F————	0	0.46
	89	RNA	-F————	0	0.83
KI-092	39	RNA	————	1.48	0.05
	86	RNA	-F————	0.41	0.13
KI-099	−44	Proviral DNA	——C————	NA	NA
	−4	RNA	-F–C————	0.04	0.06
	44	RNA	-F————	0.02	0.12
KI-102	58	RNA	————	2.11	0.45
	137	RNA	-F————	0.45	0.10
KI-126	−68	Proviral DNA	-F————	NA	NA
	19	RNA	-F————	0.01	0.06
	101	NA	NA	0	0.11
KI-134	9	Proviral DNA	-F————	NA	NA
	49	RNA	-F————	0	0.22
KI-144	−46	Proviral DNA	-F————	NA	NA
	37	RNA	-F————	0.02	2.45
	71	RNA	-F————	NA	NA
KI-150	−43	RNA	-F————	NA	NA
	21	RNA	-F————	0	0.03
	63	NA	NA	0	0.02
KI-154	−70	Proviral DNA	-F————	0.06	0.13
	77	RNA	-F————	0.01	0.35
KI-158	14	Proviral DNA	————	2.91	0.41
KI-161	−26	Proviral DNA	—I————	NA	NA
		RNA	-F-I————		
	24	Proviral DNA	-F-I————	3.94	0.05
		RNA	-F-I————		
	86	Proviral DNA	-F-I————	0.29	0.79
		RNA	-F-I————		
	52	RNA	-F————	0.71	0.66
KI-163	−81	Proviral DNA	-F————	NA	NA
		RNA	-F————		
	26	RNA	-F————	0.09	0.57
	73	NA	NA	0.02	0.59

[a] Time: Time in weeks after treatment cessation. Negative time numbers: before treatment cessation. NA, not available.

responses are different between HLA class I alleles and influenced by viral mutations in epitope regions as described elsewhere [16–22]. HLA-A*2402 is the most frequent HLA class I allele with 70% prevalence in the Japanese population [21,22]. Therefore, the majority of the study participants could be assessed by using HLA-A*2402-epitope tetramer and thus it is most beneficial to evaluate HLA-A*2402 restricted CTL responses for Japanese patients. Moreover, HLA-A*2402-restricted epitopes have been studied extensively [22] and we were able to focus on three immunodominant epitopes. This approach allowed us to find a high frequency of the escape variant Nef138-2F efficiently.

Viral mutation is one of the important mechanisms of immune escape of HIV-1 [16–23,27–29], which occurs at amino acids responsible for HLA binding, T-cell receptor recognition, or in flanking regions that affect antigen presentation. In our study Nef138-2F, which is a mutation in the immunodominant CTL epitope Nef138-10, had emerged in 5 of 12 HLA-A*2402-positive patients. Although the magnitude of Nef138-10-specific CTLs was not significantly correlated with pVLs as previous trials [15], Nef138-2F variant infection was correlated with high pVL levels in early clinical course and seemed to contribute to lower CTL response. Furthermore, we previously demonstrated the strong and weak ability of Nef138-10-specific CTL clones to suppress replication of the wild-type and 2F mutant viruses respectively [23]. In addition, although Nef138-2F-specific CTL clones suppressed the replication of both wild-type and Nef138-2F variant, their ability to suppress the replication of Nef138-2F virus was much weaker than that of Nef138-10-specific CTLs or Nef138-2F-specific CTLs against the wild-type virus replication. Furthermore, the present study demonstrated that 2F mutant appeared at the late phase in patients who had wild-type virus at the early phase. Together with these findings, frequent detection of Nef138-2F in this study strongly supports the idea that Nef138-2F is one of the escape mutations from HLA-A*2402-restricted CTLs and that Nef138-2F virus was selected by CTL pressure.

Nef138-2F mutation could occur not only by positive selection by CTLs but also by Nef138-2F-variant transmission [19–21]. Furutsuki et al. [21] reported frequent detection of

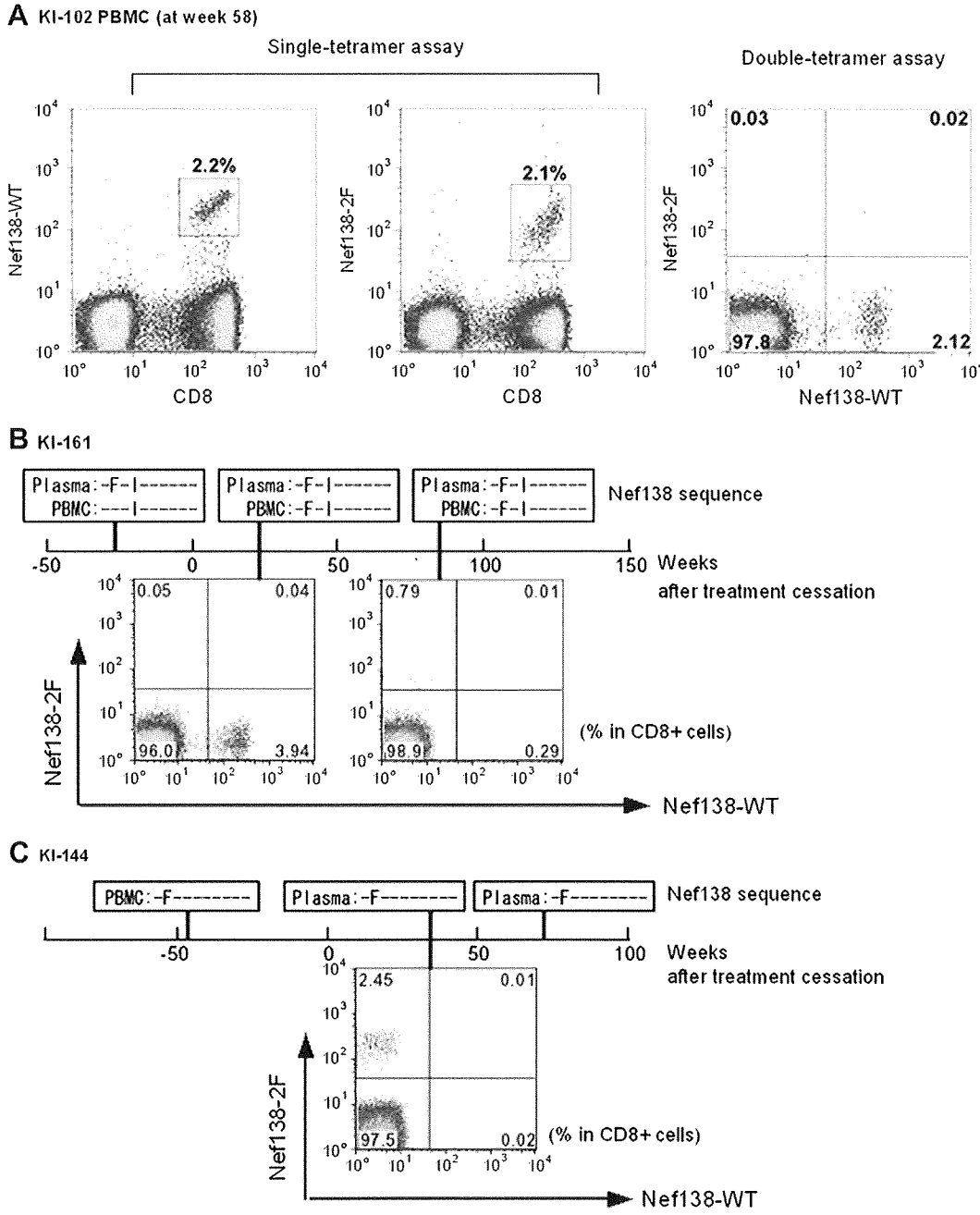

Fig. 4. Nef138-2F variant and CTL specificity. (A) PBMC of KI-102 at week 58, known to coincide with Nef138-10 wild-type infection, were assayed for wild-type Nef138-10-specific CTL (Nef138-WT-CTL) by tetramer-staining with Nef138-WT-tetramer and Nef138-2F-tetramer. The left two charts depict the results of single-tetramer-staining, showing the two tetramers stained for Nef138-WT-CTL equally (2.2% by Nef138-WT-tetramer versus 2.1% by Nef138-2F-tetramer). The right chart depicts the result of double-tetramer-staining with Nef138-WT-tetramer and Nef138-2F-tetramer, showing Nef138-WT-CTL was stained by Nef138-WT-tetramer and was differentiated from Nef138-2F-CTL. (B) Serial changes in Nef138-10 sequence and Nef138-specific-CTLs of KI-161 infected by non-Nef138-2F strain. Top: the Nef138-10 sequence; bottom charts: results of double-staining assay with Nef138-WT-tetramer and Nef138-2F-tetramer. Numbers in each quadrant represent the frequency of tetramer-positive cells among total CD8+ cells. Right lower quadrant: frequency of Nef138-WT-tetramer-positive cells; left upper quadrant: frequency of Nef138-2F-tetramer-positive cells. Note the induction of Nef138-WT-CTL and reduction in their proportion after emergence of Nef138-2F mutation. Nef138-2F-CTLs were induced after emergence of Nef138-2F mutation but their proportion was relatively lower. (C) Serial changes in Nef138-10 sequence and Nef138-specific-CTLs of KI-144 infected by Nef138-2F variant. Note the induction of Nef138-2F-CTL. Nef138-WT-CTLs were never detected throughout the study.

Nef138-2F variant in HLA-A*2402 negative Japanese patients who were infected by sexual intercourse and reversion from Nef138-2F to wild type occurred very slowly over years. These might allow horizontal spread of Nef138-2F variant. Even if the transmission of this variant in Japanese patients is very frequent, our study included the five patients who did not have this variant initially and were considered as wild-type infection, and we provided longitudinal evidence of positive selection of Nef138-2F variant under the pressure of Nef138-WT-CTLs in those.

In conclusion, our study demonstrated that early antiretroviral treatment with five series of STI did not induce a sustained immune response. A high frequency of escape mutation in the immunodominant HLA-A*2402-restricted CTLs was found, which could be one of the causes of limited immune responses by STIs.

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Successful genotype-tailored treatment with small-dose efavirenz

King and Aberg [1] recently published an excellent review of the clinical implications of population differences and genomic variations in efavirenz (EFV) treatment. They elegantly summarized the relationship between EFV concentration under standard dosage (600 mg once daily) and the genotype of cytochrome p450 2B6 (CYP2B6), a primary liver enzyme in EFV metabolism. They also highlighted the importance of CYP2B6 516 G>T SNP as a marker of individuals at risk of high EFV concentration and potential development of central nervous system (CNS) side-effects. However, it is desirable to discuss possible personalization of treatment by EFV dose modification.

As we described in our recent clinical study [2], we reduced EFV dosage in 12 patients with CYP2B6 516G>T polymorphism who were found to have extremely high EFV concentrations when treated with the standard dosage. The dosage was reduced from 600 to 400 mg in five individuals and to 200 mg in seven, and their HIV-1 load was successfully suppressed below detection limit (50 copies/ml) at these dosages. Interestingly, nine of the 12 suffered from chronic CNS-related symptoms at the standard dosage, but these improved in all nine by EFV dose reduction. An example of these patients is a 71-year-old man who reported having nightmares almost every night since starting EFV-containing antiretroviral therapy at 600 mg 3 years ago (Fig. 1). Plasma EFV concentrations were extremely high

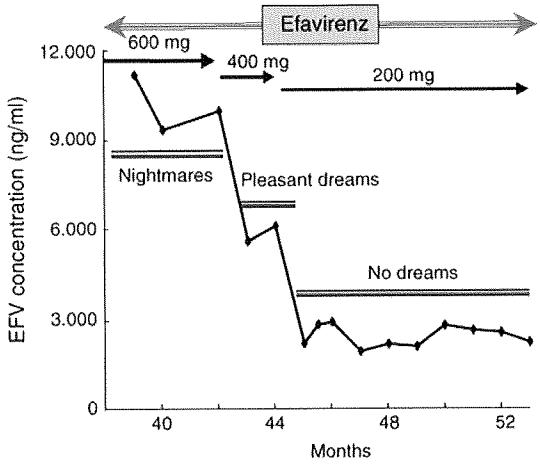

Fig. 1. Efavirenz dose reduction resulted in reduced efavirenz concentration and improved central nervous system related symptom. A CYP2B6 516T/T genotype holder reported having nightmares every night for 3 years, which disappeared after efavirenz (EFV) dose reduction.

and analysis of the 516G>T SNP showed CYP2B6 516 genotype T/T. The EFV dosage was reduced to 400 mg. This resulted in a dramatic change in dream contents from nightmares to pleasant dreams. These changes occurred although the EFV concentration remained high at 400 mg. Therefore, we further reduced the dose to 200 mg. The second reduction resulted in complete disappearance of dreams. Although he missed the dreams, the EFV concentration decreased to within the target range at 200 mg. The EFV dose has been at 200 mg for more than 2 years, and the HIV-1 load remains under detection limit.

Hasse *et al.* [3] also reported a patient with genotype CYP2B6 516T/T, who had chronic CNS symptoms and extremely high EFV concentration at 600 mg dose, but the symptoms resolved by reducing the EFV dose to 200 mg. Considered together, the above report and our study suggest that the quality of life of CYP2B6 516T/T genotype holders who suffer from CNS-related symptoms can be improved by reducing EFV dose from the standard to 400 or even 200 mg. In their review, King and Aberg [1] indicated that the cost remains an issue for identifying CYP2B6 516 genotype. However, one Japanese commercial laboratory has already developed a CYP2B6 516 genotype detection system based on the Invader assay [4], which costs only ¥8000 (~$75) per single test. Thus, the financial benefits of reducing EFV dosage should compensate for the cost of genotyping. Further large-scale studies are needed to discuss genotype-based tailored EFV treatment.

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