TABLE 1. Patient profile[a]

Patient ID	Sex	HLA type	No. of CD4 cells/μl	Viral load (copies/ml)	Sample date (mo/day/yr)	HIV subtype
A24-positive Japanese hemophiliacs						
A24-J037	M	A24/26, B35/51	207	180,000[b]	03/09/95	B
A24-J041	M	A24/26, B44/61	261	7,500[b,d]	03/09/95	B
A24-J033	M	A24/26, B46/52	27	200,000[b]	03/27/95	B
A24-J035	M	A24, B40/48	148	360,000	04/10/95	B
A24-J031	M	A24/31, B51/60	29	180,000[b]	10/23/95	B
A24-J030	M	A11/24, B13/62	3	380,000[b,d]	02/26/96	B
A24-J029	M	A24/31, B35/61	38	ND	04/01/96	B
A24-J036	M	A2 /24, B35/51	60	74,000[b]	05/13/96	B
A24-J034	M	A24, B46/52	180	74,000[b,d]	05/20/96	B
A24-J038	M	A2 /24, B51/62	356	29,000[b]	03/03/97	B
A24-J005	M	A24, B52/70	39	220,000[b]	06/19/97	B
A24-negative Japanese hemophiliacs						
NA24-J037	M	A26, B40	8	>1,600,000[b,d]	06/08/95	B
NA24-J035	M	A11/26, B54/56	342	100,000[b]	09/07/95	B
NA24-J031	M	A2/26, B51/61	521	130,000[b]	09/18/95	B
NA24-J041	M	A26, B39/54	12	700,000[b,d]	10/05/95	B
NA24-J032	M	A2/11, B46/54	1[d]	150,000[b]	11/10/95	ND
NA24-J030	M	A31/33, B44/51	363	65,000[b]	03/21/96	B
NA24-J040	M	A2/33, B17/54	101	74,000[b]	03/21/96	ND
NA24-J033	M	A26, B61	143	140,000[b]	04/18/96	B
NA24-J029	M	A11/33, B44/51	401	<10,000	07/15/96	B
NA24-J034	M	A11/33, B17/56	38	81,000[b]	08/15/96	B
NA24-J039	M	A11/26, B51/62	3	88,000[b]	09/01/97	B
NA24-J006	M	A2/26, B39/61	335	9,200	10/30/00	B
A24-positive Japanese infected through USI						
A24-J006	M	A2/24, B7/54	212	33,000	09/19/97	B
A24-J007	M	A24/26, B17/56	103	120,000	11/06/97	B
A24-J009	M	A24, B48/52	278	4,500	01/19/98	B
A24-J010	M	A24, B52	393	18,000	03/09/98	B
A24-J024	M	A24, B35/61	274	110,000	10/27/98	B
A24-J012	M	A24/26, B46/60	253	24,000	07/19/99	B
A24-J013	M	A24/26, B35/48	168	15,000	9/20/99	B
A24-J016	M	A11/24, B7/55	245	150,000	05/15/00	B
A24-J017	M	A1/24, B54/70	255	70,000	10/17/00	B
A24-J018	M	A24/31, B37/61	185	8,300	01/04/01	B
A24-J025	M	A24, B51/52	282	130,000	06/07/01	B
A24-J023	M	A2/24, B51/54	856[d]	17,000[d]	08/06/01	B
A24-J021	M	A2/24, B46/52	344	35,000	11/26/01	B
A24-J026	M	A2/24, B13/51	381	110,000	11/28/01	B
A24-negative Japanese infected through USI						
NA24-J025	M	A2/31, B51/61	352	18,000[b]	03/23/95	B
NA24-J023	M	A11/26, B35/51	23	5,000[b]	04/01/96	ND
NA24-J021	M	A26, B52/54	9	44,000	08/04/97	B
NA24-J018	M	A2, B39/60	378	72,000	04/06/98	B
NA24-J017	M	A11/31, B51/56	197	72,000	04/16/98	B
NA24-J016	M	A3/31, B51/58	257	200,000	05/25/98	B
NA24-J015	M	A2/26, B51/62	543	13,000	06/26/98	B
NA24-J012	M	A31, B13/51	268	26,000	10/19/98	B
NA24-J011	M	A2, B55/60	408	12,000	10/22/98	B
NA24-J010	M	A2/26, B51/61	206	16,000	12/17/98	B
NA24-J009	M	A2, B52/60	115	850,000	05/24/99	B
NA24-J008	M	A11/33, B44/60	312	2,600	07/08/99	ND
NA24-J007	M	A26, B7/52	396	450	08/09/00	B
NA24-J005	M	A2/31, B48/52	604	17,000	01/18/01	B
NA24-J003	M	A31/33, B44/51	308	20,000	06/04/01	B
NA24-J002	M	A2/33, B44/46	496	14,000	09/27/01	ND
A24 positive Australian infected through USI						
A24-A001	M	A3/24, B7	255	38,000	08/16/96	ND
A24-A002	M	A24/30, B13	598	21,700	03/22/01	B
A24-negative Australian infected through USI						
NA24-A007	M	A2/3, B7	704	ND[c]	11/02/95	B
NA24-A005	M	A1/3, B8/70	620	7,700	05/26/96	B
NA24-A013	M	A32, B13/64	851	23,200	09/28/98	B
NA24-A008	M	A2/3, B39/44	543	52,836	01/04/99	B
NA24-A003	M	A2, B18/62	575	19,400	11/06/99	B
NA24-A006	M	A3/26, B18/27	594	18,200	04/13/00	B

[a] ND, not determined.
[b] Data were obtained by Branch DNATM version 1.0.
[c] Nearest data were 17,000 with CD4 counts of 638.
[d] Nearest data were within 6 months of sample collection.

A

A24-positive Japanese hemophiliacs

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV
A24-J041	---------V	-F--------	---------M
A24-J033	-----E---T	-F-------Y	-------D--
A24-J031	-H-------T	-F--------	-
A24-J030	---------T	-F--C-----	----------
A24-J034	---------T	-F--------	-----DQ-Q-
A24-J038	----------	----C-----	-----D-D--
A24-J005	-D/E-------T	-F--------	-
A24-J029	---------V/T	-F--------	--------Q-
A24-J037	--C------T	-F--------	-----D----
A24-J035	---------T	-F--------	----------
A24-J036	--C------T	-F--------	----------

A24-negative Japanese hemophiliacs

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV
NA24-J037	----------	----------	----------
NA24-J035	----------	----------	----M-----
NA24-J031	----------	----------	--------G/E-V/I
NA24-J041	----------	----------	--------DE
NA24-J032	----------	----------	----M-----
NA24-J030	--S------V	----C-----	----------
NA24-J040	----------	----------	---------I
NA24-J033	----------	----------	-L/V-------
NA24-J029	-H--------	----------	--------D-
NA24-J034	----------	----------	----V/L-----
NA24-J039	----------	----C-----	-----D-D--
NA24-J006	---------V	----C-----	-----E----

B

A24-positive Japanese infected through USI

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV
A24-J006	---------V	-F--------	-----S/D--Q-
A24-J007	---------T	-F--C-----	----A---E-
A24-J009	---------T	-F--------	-
A24-J010	---------T	-F--------	-------QR-
A24-J012	---------T	-F--------	-------D--
A24-J013	---------T	-F--------	-----D-DQ-
A24-J016	-D-------V	----C-----	-----DQD--
A24-J017	-D-------T	-F--C-----	---------I
A24-J018	---------T	-F--------	---------I
A24-J023	---------T	-F--------	----L--GEA
A24-J021	---------T	-F--------	-----D-DQ-
A24-J024	---------T	-F--------	-----D-D--
A24-J025	-D-------T	-F--------	-----DQDQ-
A24-J026	---------T	-F--------	-------KQ-

A24-negative Japanese infected through USI

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV	
NA24-J025	-H-------V	----C-----	-----D-D/AQ-	
NA24-J023*	---------T	-Y/W/F--------	---------I	Y 2/11,F 3/11,W 5/11
NA24-J021	----------	----------	-----N--Q-	
NA24-J018*	---------T	-Y/F--C-----	----------	Y 8/10,F 2/10
NA24-J017*	---------T	-Y/F--------	----L-----	Y 5/13,F 8/13
NA24-J016	---------V	----------	----L---Q-	
NA24-J015	---------T	-F--------	-----D-DQ-	
NA24-J012	-H/QS------T	----------	-----D-DQ-	
NA24-J011	---------T	-F--------	-------NQ-	
NA24-J010	-	----------	-	
NA24-J009	---------T	-F--------	-------NQ-	
NA24-J008	-D-------T	-F--------	----L---Q-	
NA24-J007	---------T	-F--------	-------NQ-	
NA24-J005	-G/D-------T	-F--------	-----DQDQ-	
NA24-J003	-H--------	----------	-----DQ---	
NA24-J002	-Q/HG-------	----------	-----D-DQ-	

C

A24-positive Australian infected through USI

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV
A24-A001	---------T	-F--------	----------
A24-A002	---------T	-F--------	----M-----

A24-negative Australian infected through USI

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV
NA24-A007	---------V	----------	-
NA24-A005	---------V	----------	----------
NA24-A013	----------	----------	----------
NA24-A008	-H--------	----------	----M-P/Q---
NA24-A003	-H--------	----------	-----D-D--
NA24-A006	----------	----C-----	--------E-

FIG. 1. Nef138-10 epitope and its flanking sequences. Amino acid sequences deduced from the direct DNA sequencing of Nef138-10 CTL epitope and both flanking regions are presented. Wild-type sequences (HIV-1 strain SF2) are presented on the top. Dashes indicate the same amino acid as that of the wild type. Sequence substitutions are presented by single amino acid characters. Where a mixture of two or three amino acids was plausible, two or three amino acids were shown together separated by a shill. A single dash indicates that the sequences could not be determined by ambiguities. (A) Sequences from A24-positive and -negative Japanese hemophiliacs. (B) Sequences from A24-positive and -negative Japanese patients infected through USI. Asterisks indicate samples for which sequence ambiguities were found by direct sequence analysis. We cloned these PCR fragments into the pGEM-T vector and sequenced each 10 to 13 colonies. All amino acid sequences are indicated. (C) Sequences from A24-positive and -negative Australians infected through USI.

frequent among the Japanese population, which has a higher prevalence of HLA-A*2402.

Nef138-10(2F) accompanied a particular amino acid substitution in the N-terminal flanking region. We detected an I-to-T substitution at the −1 position (−1T) in 32 flanking sequences out of 34 accompanying Nef138-10(2F) sequences (94%), while others were two I-to-V substitutions (Fig. 1). The −1T substitution was quite unusual in the flanking region of the wild-type Nef138-10 CTL epitope in our cohort (Fig. 1).

Reversion of CTL escape mutants. Since three acutely infected A24-positive patients (A24-J023, A24-J024, and A24-J025) had Nef138-10(2F) in their earliest plasma samples available, we could not demonstrate the evolution of Nef138-10(2F) from the wild type under the selective pressure of HLA-A*2402 (data not shown). However, we could analyze serial samples from chronically infected A24-negative patients who had been followed without treatment over years. All the 12 cloned sequences in the earliest plasma samples available from NA24-J015 had F at the second position; however, 11 out of 11 clones displayed wild-type sequence within a year (Fig. 2A). It is interesting that the −1T substitution in the flanking region was present for at least a further two years before reverting to the wild type (Fig. 2A). In another chronically infected A24-negative patient (NA24-J018), we observed that the proportion of Nef138-10(2F) decreased gradually but persisted in the plasma for almost two years after the start of the follow-up (Fig. 2B). This patient had a T-to-C substitution at the fifth position with [Nef138-10(2F5C)] or without [Nef138-10(5C)] a substitution at the second position (Fig. 2B). Interestingly, the ratio of Nef138-10(2F5C) to Nef138-10(5C) decreased as time went by (Fig. 2B), suggesting that Nef138-10(5C) is more stable than Nef138-10(2F5C). Actually, we observed Nef138-10(5C) in both A24-positive and -negative patients (Fig. 1).

In order to elucidate the higher stability of the 5C rather than the 2F substitution, we examined the codon usage at these positions (data not shown). The wild-type codon for the second tyrosine (Y) residue in Nef138-10 was coded by TAT or TAC in 23 (77%) and 12 (40%) out of 30 patients, respectively. Five patients (17%) had a mixture of TAT and TAC for the codon (data not shown). Mutated nucleotide triplet TTT or TTC was responsible for the Y-to-F amino acid substitution in 27 (80%) and 9 (26%) out of 34 patients, respectively (data not shown).

A

	Sample Date	nef138-10			
Patient ID	M/D/Y	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV	cloning
NA24-J015	06/26/98	---------T	-F--------	-----D-DQ-	direct
		---------T	-F--------	-----D-DQ-	11/12
		---------T	-F-----R--	-----D-DQ-	1/12
NA24-J015	06/07/99	---------T	-Y--------	-----D-DQ-	direct
		---------T	-Y--------	-----D-DQ-	9/11
		-R-------T	-Y--------	-----D-DQ-	1/11
		---------T	-Y--------	--I--D-DQ-	1/11
NA24-J015	03/09/00	---------T	-Y--------	-----D-DQ-	direct
NA24-J015	04/16/01	---------T	-Y--------	-----D-DQ-	direct
NA24-J015	01/16/03	----------	-Y--------	-----D-DQ-	direct
		----------	-Y--------	-----D-DQ-	10/10

B

	Sample Date	nef138-10			
Patient ID	M/D/Y	WQNYTPGPGI	RYPLTFGWCF	KLVPVEPEKV	cloning
NA24-J018	04/08/96	---------T	-F--C-----	--------Q-	direct
		---------T	-F--C-----	--------Q-	7/11
		---------P	-F--C-----	--------Q-	3/11
		---------P	-Y--C-----	--------Q-	1/11
NA24-J018	06/02/97	---------T	-F/Y--C-----	--------Q-	direct
		---------T	-F--C-----	--------Q-	7/13
		---------T	-Y--C-----	--------Q-	3/13
		---------A	-Y--C-----	--------Q-	1/13
NA24-J018	04/06/98	---------T	-Y/F--C-----	----------	direct
		---------T	-Y--C-----	--------Q-	6/10
		---------T	-F--C-----	--------Q-	2/10
		---------T	-Y--C-----	----------	2/10

FIG. 2. Serial Nef138-10 epitope and its flanking sequences in two A24-negative patients. Data are shown as described in the legend to Fig. 1. Fractional numbers in the right-most column indicate clone numbers with the sequences shown in the numerator and total clone numbers sequenced shown in denominator. "Direct" indicates the result of direct sequencing. (A) Patient NA24-Jo15. (B) Patient NA24-J018.

In two patients (6%) Nef138-10(2F) was coded by a mixture of HIV-1 using TTT and TTC for the codon. It appeared that at least one point mutation was necessary for the Y-to-F amino acid substitution. The wild-type codon for the fifth threonine (T) residue in Nef138-10 was coded by ACC or ACT in 49 (98%) and 2 (4%) out of 50 patients. One patient (2%) had a mixture of ACC and ACT. Mutated nucleotide triplet TGC or TGT was responsible for the T-to-C amino acid substitution in 5 (45%) and 6 (55%) out of 11 patients, respectively (data not shown). It appeared that at least two nucleotides had to be mutated for the T-to-C amino acid substitution, although we could not exclude the possibility of a three-nucleotide mutation for the amino acid substitution. Therefore, a Y-to-F amino acid substitution, or vice versa, at the second position required less nucleotide mutations than did the T-to-C substitution at the fifth position.

Peptide-based analysis of Nef138-10 and its variants. We measured the binding affinities of Nef138-10 and its variants to HLA-A*2402 (Fig. 3). Although a Y-to-F substitution occurred at the amino acid crucial for peptide affinity with the binding groove, Nef138-10(2F) binding to the HLA-A*2402 heavy chain was essentially preserved. However, the acquisition of a T-to-C substitution at the fifth position, such as Nef138-10(2F5C) and Nef138-10(5C), greatly reduced the binding affinity (Fig. 3). A functional avidity assay in which PBMCs from five patients were used confirmed the results of the binding assay (Fig. 4). Namely, the patients' PBMCs recognized Nef138-10(2F) at a very low concentration (one-half maximum response <1 nM) and had equivalent or even higher functional avidity than did the wild-type peptide. On the contrary, patients' PBMCs showed very low functional avidity against Nef138-10(2F5C) and Nef138-10(5C) (one-half maximum response >100 nM).

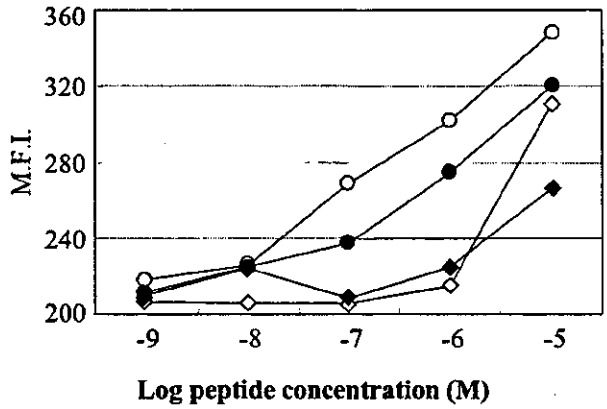

FIG. 3. Binding of the wild-type and mutant peptides to HLA*2402 molecules. Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay. Symbols: ○, wild type; ●, 2F; ◇, 5C; ◆, 2F5C. M.F.I., mean fluorescence intensity.

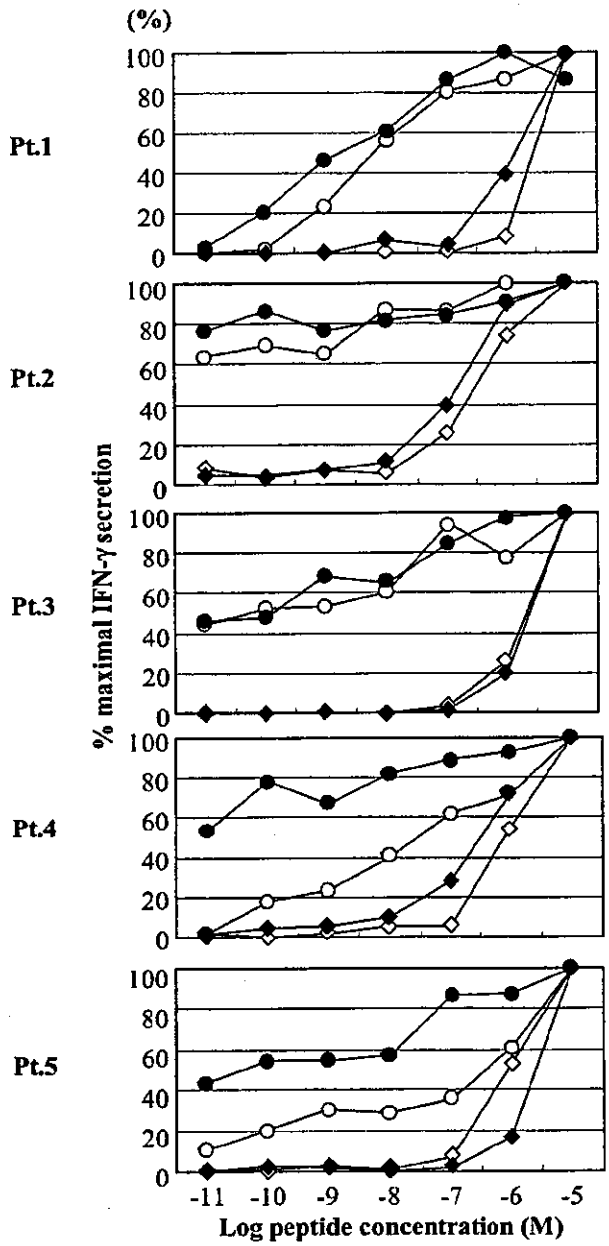

FIG. 4. Functional avidity assay. The reactivity of peptide-specific cells in PBMCs from five patients against log-fold dilutions of peptide was determined. Symbols: ○, wild type. ●, 2F; ◇, 5C; ◆, 2F5C.

Epitope presentation from native Nef protein. Strong selection for Nef138-10(2F) in the presence of CTLs with high in vivo functional avidity against the peptide prompted us to examine the processing and presentation of the Nef138-10 CTL epitope from the native protein. Native Nef proteins containing wild-type or variant CTL epitopes were expressed in an HLA-A*2402-positive-T-cell line (KWN-T4) via SeV. CTL epitope presentation was examined by two CTL clones established from A24-positive patients outside these cohorts. Although the two CTL clones were established by stimulation with the wild-type peptide (Nef138-10), they killed the target cells pulsed with Nef138-10(2F) peptides almost as well as the wild type (Fig. 5A and B). Both CTL clones efficiently killed the target cells expressing either wild-type Nef or Nef with −1T substitution in the flanking region (−1T2Y5T). However, these CTL clones failed to kill the target cells infected with vectors expressing Nef138-10(2F) with (−1T2F5T) or without (−1I2F5T) the −1T substitution in the flanking region. As expected, the CTL clones did not kill the target cells infected with a vector coding Nef138-10(2F5C), a nonbinding mutant (−1I2F5C) (Fig. 5A and B). Western blot analysis revealed that Nef proteins with wild-type or variant CTL epitopes were expressed abundantly in the target cells. Taken together, these data indicate that a Y-to-F substitution within the CTL epitope itself but not the −1T substitution in the flanking region resulted in the poor antigen presentation against CTL, which resulted in the escape.

DISCUSSION

We showed a significantly higher prevalence of a stereotypic amino acid substitution [Nef138-10(2F)] at an A24-restricted CTL epitope in Nef among A24-positive Japanese hemophiliacs compared with A24-negative counterparts. The origin of their HIV-1 infection was from the plasma collected and processed in Western countries where HLA-A*2402 was less prevalent (19). It is inferred that Nef138-10(2F) might be rare in a population where HLA-A*2402 is not prevalent but that it has a selective advantage in the presence of HLA-A*2402. Our findings with Australians are consistent with this notion. Although we examined only two HIV-1-infected A24-positive Caucasian Australians, both had Nef138-10(2F). On the other hand, Nef138-10(2F) was rare in A24-negative Australians. Japanese and Australians are distinctly different in the frequency of HLA-A*2402 within their respective populations (allele frequency of HLA-A24 is 35.1 and 7.8%, respectively) (19). Nef138-10(2F) was also positively selected among Japanese patients who were infected through USI. Interestingly, we detected Nef138-10(2F) frequently among A24-negative Japanese who were infected through USI. The result suggests that HIV-1 that went through selective pressure by HLA-A*2402 is actually circulating among the Japanese population because of the high prevalence of HLA-A24. Although we showed the reversion of Nef138-10(2F) to the wild type, it occurred very slowly over years, allowing the horizontal spread via sexual contact. In this study, we showed that HIV-1 with Nef138-10(2F) is actually a CTL escape mutant. Although the stereotypic Y-to-F substitution occurred at an anchor residue, Nef138-10(2F) peptide did bind to HLA-A*2402 heavy chain with almost the same efficiency as did the wild type (Fig. 3). This result is consistent with the algorithm prediction of the published binding motif (http://hiv-web.lanl.gov/content/immunology/motif_scan/motif.html). When native Nef proteins with or without a substitution were overexpressed in the A24-positive target cells via SeV, the Y-to-F substitution at the second position of the CTL epitope virtually abolished the killing by the CTL clones. The substitution in the flanking region did not affect the killing substantially. Therefore, the mechanism for the CTL escape appeared to reside in the processing of native Nef proteins and subsequent antigen presentation rather than HLA binding. A proteosomal cleavage

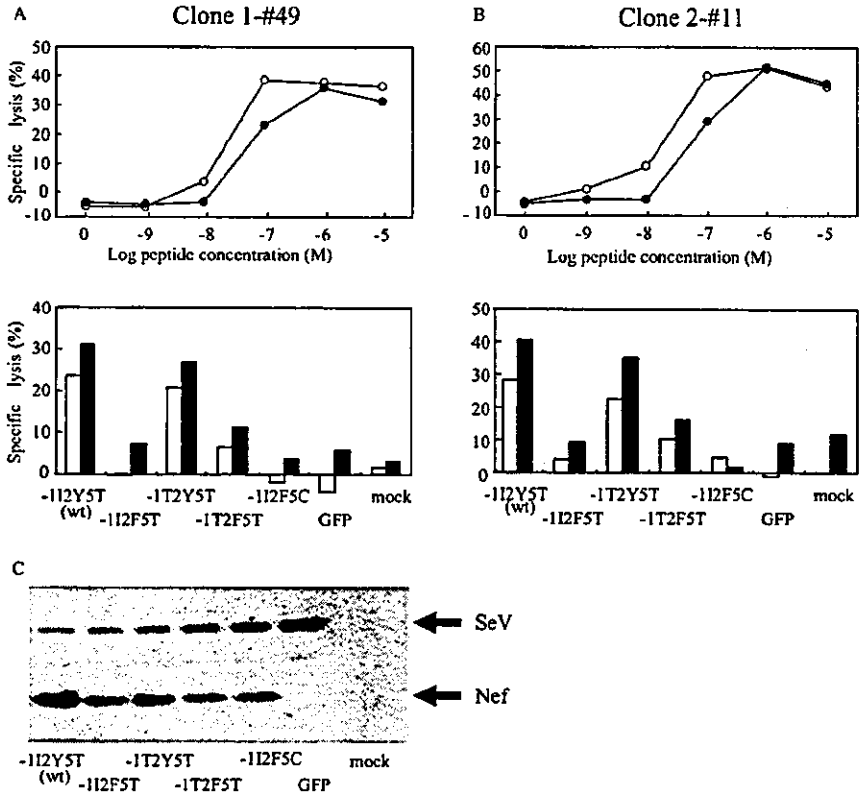

FIG. 5. Killing activity of clone 1-#49 (A) against KWN-T4 target cells pulsed with log-fold dilutions of peptide (top) and expressing native Nef proteins containing wild-type sequences (−1I2Y5T), a Y-to-F substitution at the second position of the CTL epitope (−1I2F5T), an I-to-T substitution at the −1 flanking position (−1T2Y5T), double substitutions at the −1 and second positions (−1T2F5T), and double substitutions at the second and fifth positions (−1I2F5C) (bottom). The effector-versus-target ratio was 1:1 (□) or 2.5:1 (■) in panel A and 1:1 (□) or 4:1 (■) in panel B. Killing activity against KWN-T4 cells infected with control vector expressing green fluorescent protein (GFP) and mock infected (mock) are also shown. (C) Western blot analysis of intracellular expression of various Nef mutants in KWN-T4 target cells. KWN-T4 target cells expressing native Nef proteins containing wild-type sequences (−1I2Y5T), a Y-to-F substitution at the second position of the CTL epitope (−1I2F5T), an I-to-T substitution at the −1 flanking position (−1T2Y5T), double substitutions at the −1 and second positions (−1T2F5T), and double substitutions at the second and fifth positions (−1I2F5C) were examined. KWN-T4 cell lysates infected with control vector expressing GFP and mock infected are also shown. An aliquot (3 μg) of the same KNW-T4 target cells used for the killer assay in the upper panel was used for the Western blot. Symbols: ○, Nef138-10; ●, Nef138-10(2F).

prediction program, NetChop (23), suggested the possibility that the Y-to-F substitution in the second position creates a new cleavage site at the fifth T residue in the CTL epitope. Proteolytic cleavage within the epitope could be the cause of poor antigen presentation.

Although we could not show the process of positive selection for Nef138-10(2F), Nef138-10(2F5C), and Nef138-10(5C), the high prevalence of Nef138-10(2F) in A24-positive patients and the reversion in A24-negative patients suggested that one point mutant, Nef138-10(2F), was selected first, and then two or three point mutants, Nef138-10(2F5C), evolved. Once the T-to-C amino acid substitution at the fifth position is acquired, the binding capacity of the CTL epitope to the HLA-A*2402 heavy chain is abolished (Fig. 3), and the Y-to-F substitution at the second position may become dispensable even in the presence of HLA-A*2402.

In our cohort of patients, Nef138-10(2F) accompanied a −1T substitution in the flanking region very frequently. We observed sequential reversion in the CTL epitope and flanking region at least in one patient with an A24-negative background. As of 11 October 2003, the HIV-1 sequence database showed that the 2F substitution (74 sequences) accompanied the −1T substitution frequently (64.9%) but accompanied the wild-type residue (I) only rarely (9.5%). On the other hand, the wild-type residue (Y) in the second position of the CTL epitope (195 sequences) accompanied wild-type (I) residue more frequently (57.4%) than the −1T substitution (20.5%). Although the function of the region surrounding Nef138-10 has not been elucidated, there seems to be a compensation between these two residues.

In simian immunodeficiency virus infection, CTLs with high functional avidity select for escape variants (29). However, we found CTLs with high functional avidity not only against the wild type but also against Nef138-10(2F) in five patients studied. It is not known how these CTLs against Nef138-10(2F) are maintained in vivo. Very recently, new insights into the exogenous pathway for antigen presentation to CTLs have been elucidated (15, 17). Cross presentation by professional antigen-

presenting cells such as dendritic cells may be responsible. Our study underlines the difficulties in evaluating the effective CTL responses in vivo by CTL assays in which peptides are used, such as ELISPOT.

For example, a CTL escape variant of Epstein-Barr virus was demonstrated in a highly A11-positive population in New Guinea (9). HLA-restricted CTL responses appear to be driving HIV-1 evolution at a population level (20). As far as we know, this is the first direct demonstration of horizontal transmission of CTL escape mutants of HIV-1 at a population level. We previously reported stereotypic amino acid substitutions in HIV-1 at some CTL epitopes restricted by HLA-B35 (21). Stereotypically selected HIV-1 may become dominant through transmission where certain HLA types are highly prevalent. Recently, a rare HLA supertype was shown to have a selective advantage for the prognosis of HIV-1 infection (34). In a population with less diverse HLA types, such as that of Japan, HLA types may have a large impact on HIV-1 evolution and escape. Our study may prove to have important implications for understanding viral pathogenesis and vaccine development.

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Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy

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Protease inhibitors (PIs) such as nelfinavir (NFV) suppress HIV replication. PIs are substrates of P-glycoprotein (P-gp), the product of the multidrug-resistance-1 (*MDR1*) gene. Three single-nucleotide polymorphisms (SNPs) are present in exons of the *MDR1* gene: *MDR1* 1236, *MDR1* 2677 and *MDR1* 3435. We speculated that these genetic polymorphisms affected PI concentration in the cell. To verify this hypothesis, we first genotyped these SNPs in 79 Japanese patients by the SNaPshot method and found incomplete linkage disequilibrium between the SNPs. Because the SNP at *MDR1* 3435 has been reported to be associated with P-gp expression, we evaluated the effect of that SNP on the export of NFV from HIV-positive patients' lymphoblastoid cell lines by measuring time-dependent decrease in the amount of intracellular NFV by high-performance liquid chromatography. We found the intracellular concentration of NFV in lymphoblastoid cell lines (LCLs) with the homozygous T/T genotype at *MDR1* 3435 were higher than that with C/C genotype with statistical significance. This suggests that the activity of P-gp in patients' LCL cells with the *MDR1* 3435 T/T genotype was lower. In a retrospective study we evaluated the effect of the SNPs on CD4 cell count recovery in response to antiretroviral treatment with PIs, and obtained statistically significant evidence that suggested marginal association of the SNP at *MDR1* 1236 but not at *MDR1* 2677 or *MDR1* 3435. As *in vitro* results were not consistent with the clinical evaluation, clinical importance of *MDR1* genotyping for antiretroviral therapy remains to be investigated in a larger, case-controlled study.

Introduction

Antiretroviral therapy with HIV protease inhibitors (PIs) in combination with reverse transcriptase inhibitors dramatically improved the prognosis of patients infected with HIV-1. However, some patients fail to achieve the maximal virological suppression. We speculate that such failure is partly because PIs do not accumulate in lymphocytes in their active free forms in a concentration high enough to inhibit viral replication [1,2], although the intracellular active PI levels have, to the best of our knowledge, not yet been determined. The activity of P-glycoprotein (P-gp), the product of the multidrug resistance-1 (*MDR1*) gene, appears to affect intracellular PI concentration, because PIs such as nelfinavir (NFV) are substrates of P-gp [2]. P-gp is a glycosylated membrane protein belonging to the ATP-binding cassette superfamily of membrane transporters. P-gp is expressed in many tissues and cell types including intestinal epithelial cells and lymphocytes, where it acts as an energy-dependent exporter [3–9]. The *MDR1* is polymorphic and at least three single-nucleotide polymorphisms (SNPs) have been identified in the exons in a healthy Japanese population [10] as well as in other ethnic groups [6]. *MDR1* 1236 and *MDR1* 3435 are silent mutations in exons 12 and 26 [3,11], respectively, whereas *MDR1* 2677 is a substitution mutation in exon 21 [11]. Reportedly, the SNP at *MDR1* 3435 is associated with the amount and activity of P-gp protein both *in vitro* and *in vivo* [3,12]. In addition, individuals with the T/T genotype at *MDR1* 3435 were found to express less P-gp in lymphocytes and in intestinal epithelial cells [3,13] and showed lower efflux of rhodamine from natural killer (NK)

cells than those with the C/C genotype [13]. According to these observations, *MDR1* polymorphisms seem to affect the intracellular PI concentration and the outcome of antiretroviral treatment. However, the role of *MDR1* 3435 SNP in the response to antiretroviral therapy is still controversial [12,14].

The objective of this study was to evaluate the effect of three *MDR1* SNPs on the intracellular concentrations of NFV and to evaluate the impact of those SNPs on virological and immunological response to antiretroviral treatment, including NFV and PIs. We genotyped the SNPs in 79 Japanese patients and compared the velocity of NFV efflux among selected patients' lymphoblastoid cell lines (LCLs) with different *MDR1* 3435 genotypes. We also analysed the viral loads and CD4 cell counts after initiation of antiretroviral treatment with prescriptions with PIs including NFV in 21 patients.

Materials and methods

Patients

A total of 79 HIV-positive Japanese patients were enrolled in this study. These patients attended a hospital AIDS clinic at the Institute of Medical Science, University of Tokyo (IMSUT). The patients provided their written informed consent to participate in the study and to supply blood samples for DNA analysis and cell culture. Of the 79 patients, 21 receiving highly active antiretroviral therapy (HAART) including PIs were divided into three groups: 11 patients receiving HAART with NFV, four patients receiving HAART with indinavir (IDV) and six patients receiving HAART with saquinavir (SQV) or lopinavir/ritonavir (LPV/RTV). CD4 cell counts and HIV-RNA of plasma were analysed for 9 months after the initiation of the antiretroviral treatment. The study has been approved by the ethics committee of IMSUT.

Single-nucleotide polymorphisms

We typed three single-nucleotide polymorphisms (SNPs) at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) by polymerase chain reaction (PCR) followed by ABI PRISM SNaPshot Multiplex Kit (PE Biosystems, Foster City, Calif., USA) [15]. Information on primers and conditions for PCR was obtained at http://snp.ims.u-tokyo.ac.jp [10].

Cells and determination of uptake and efflux of NFV

Peripheral blood mononuclear cells (PBMCs) were separated from patients' whole blood with Ficoll-Conray gradient centrifugation. LCLs were obtained by transforming PBMCs with Epstein-Barr virus (EBV), which was obtained from cell-free supernatants of EBV-producing B95–8 cell lines [16]. LCLs were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 10% heat-inactivated fetal calf serum.

To determine the time course of NFV uptake into LCL cells, LCL cells (1×10^6/10 ml, counted with a haematocytometer) were incubated at 37°C in a medium containing 10 μM NFV. Cells were harvested by centrifugation at 1500 ×*g* for 5 min at 4°C and immediately frozen at –80°C until high-performance liquid chromatography (HPLC) analysis. To determine the velocity of NFV efflux from LCL cells, these patients' LCL cells were incubated at 37°C in a medium containing 10 μM NFV for 3 h. The cells were then quickly washed twice with 10 ml ice-cold phosphate-buffered saline and cultured in 10 ml NFV-free medium for up to 3 h. After an interval, aliquot cells were harvested by centrifugation at 1500 ×*g* for 5 min at 4°C and immediately frozen at –80°C until HPLC analysis.

Reverse transcription-PCR (RT-PCR)

For quantification of *MDR1* transcript, RNA from 1×10^7 LCL cells was isolated using Trizol reagents (Invitrogen Corp, Carlsbad, Calif., USA). First strand cDNA was obtained by using ReverTra Ace (Toyobo, Osaka, Japan) with 1 μg of total RNA. cDNA was subjected to PCR. Information on primers and conditions for PCR was obtained as previously described [17]. We used human glyceraldehyde 3-phosphate dehydrogenase mRNA as a positive control.

Determination of intracellular concentration of NFV by HPLC

The patients' frozen LCL cells were extracted with 1.5 ml of ethanol. The extracts were then clarified by centrifugation at 2050 ×*g* for 10 min at 4°C. The ethanol extracts were evaporated at 30°C and dissolved in 180 μl of mobile phase, which was a mixture of phosphate buffer (containing 50 mM KH_2PO_4 and 50 mM Na_2HPO_4; pH 5.63) and acetonitrile (1:1, v:v) [18]. The amounts of NFV were measured using a Sensyu Pack ODS C_{18} column (5 μm particle size; 150×4.6 mm, Sensyu Scientific Co, Tokyo, Japan) at a flow rate of 1.5 ml/min by HPLC (Shimadzu Co, Tokyo, Japan). The UV detection wave length was 220 nm and efavirenz (EFV) was used as an internal standard. The lower limits of detection and quantification were 20 ng (30.1 pmole)/10^6 cells, and the calibration range was 20–2000 ng (30.1–3010 pmole/10^6 cells).

Results

We typed the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) in DNA samples from 79 HIV-positive Japanese patients

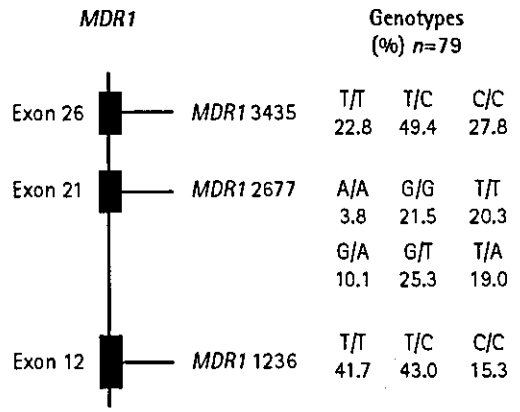
(Figure 1). We found that it was consistent with the Hardy–Weinberg principle (Tables 1 and 2). Furthermore, in all possible two-way comparisons of the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21, excluding the genotypes containing G) and *MDR1* 3435 (exon 26), we found significant linkage disequilibrium between *MDR1* 2677 A (T) and *MDR1* 1236 C (T), *MDR1* 2677 A (T) and *MDR1* 3435 C (T), and *MDR1* 1236 C (T) and *MDR1* 3435 C (T), respectively.

Reportedly, *MDR1* 3435 T/T genotype was associated with lower expression of P-gp in leukocytes [13] so we hypothesized that the genotype was also associated with slower cellular export of NFV in patients' lymphocytes. To investigate this, we first established LCLs by immobilizing selected patients' PBMCs with EBV. We selected eight patients' LCLs with *MDR1* 3435 C/C (n=4) and T/T (n=4) and verified similar levels of *MDR1* in these LCLs by RT-PCR (Figure 2). We observed little variation in *MDR1* transcripts.

We found that uptake of NFV was rapid into LCLs reaching a steady-state within 5 min (Figure 3). We studied eight patients' LCLs with *MDR1* 3435 T/T and *MDR1* 3435 C/C to compare the steady-state intracellular concentration of NFV after 3 h incubation in a medium containing 10 μM NFV. The intracellular concentrations of NFV in LCLs with *MDR1* 3435 T/T and C/C genotypes were 2593 μM and 2411 μM, respectively (n=4), with no statistical difference. We calculated these values by hypothesizing that the LCLs are ideal spheres (10 μm diameter) and that NFV distributes uniformly in the cell.

We then compared NFV efflux from those LCLs with different genotypes at *MDR1* 3435. Before measuring export of NFV, LCLs were cultured with NFV to a saturated level. These NFV-loaded cells were transferred to NFV-free medium and cultured for 3 h with intermittent sampling of cell aliquots. We compared the efflux of NFV from the eight patients' LCLs with *MDR1* 3435 T/T and C/C (n=4 each), which had been verified to express *MDR1* mRNA by

Figure 1. Frequency of SNPs in *MDR1*



The SNPs at *MDR1* 1236, *MDR1* 2677 and *MDR1* 3435 were typed by the SNaPshot method. Genotype frequencies at each site are shown as percentage among 79 HIV-infected Japanese patients. The thin vertical line at left represents the *MDR1* gene on human chromosome 7. The closed boxes represent exons 12, 21 and 26.

Table 1. Hardy–Weinberg principle at *MDR1* 1236 (n=79)

	T/T	T/C	C/C
Observed number of patients	33	34	12
Expected number of patients	31.7*	36.7†	10.6‡

p: Frequency for the T allele $\frac{33\times2+34}{2\times79}=0.633$

q: Frequency for the C allele 1−p=0.367

*$79\times p^2=31.7$

†$79\times2pq=36.7$

‡$79\times q^2=10.6$

Table 2. Hardy–Weinberg principle at *MDR1* 3435 (n=79)

	T/T	T/C	C/C
Observed number of patients	18	39	22
Expected number of patients	17.8*	39.4†	21.8‡

p: Frequency for the T allele $\frac{18\times2+39}{2\times79}=0.475$

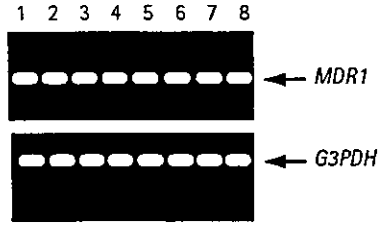
q: Frequency for the C allele 1−p=0.525

*$79\times p^2=17.8$

†$79\times2pq=39.4$

‡$79\times q^2=21.8$

Figure 2. *MDR1* mRNA expression in LCLs

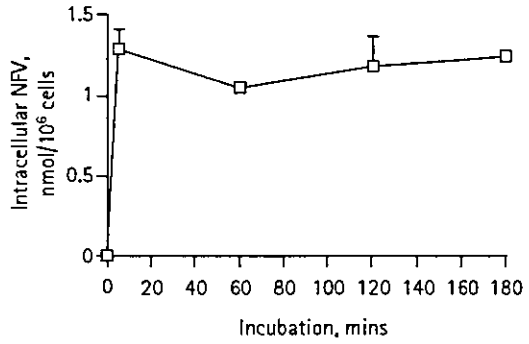


We selected eight patients' LCLs with *MDR1* 3435 C/C (lanes 1–4) and T/T (lanes 5–8) and measured the expression of *MDR1* mRNA. Total cellular RNA from LCLs was subjected to RT-PCR with primer sets for *MDR1* and *G3PDH* transcripts. Aliquots were subjected to agarose gel electrophoresis. The genotypes at *MDR1* 1236, 2677 and 3435: lanes 1 and 2, (T/T, G/G, C/C); lane 3, (T/C, G/A, C/C); lane 4 (C/C G/A C/C); lane 5 (T/T, G/T, T/T); and lanes 6–8 (T/T, T/T, T/T).

RT-PCR (Figure 2). The concentration of intracellular NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with C/C genotype at 120 min and 180 min. This difference was statistically significant (P=0.04 and 0.02, respectively, Mann–Whitney U-test, Figure 4). This meant the NFV efflux in patients' LCL cells with the *MDR1* 3435 T/T genotype was slower than that with C/C genotype. Thus, we suspect the activity of P-gp in patients' LCLs with the *MDR1* 3435 T/T genotype is lower than that with the C/C genotype.

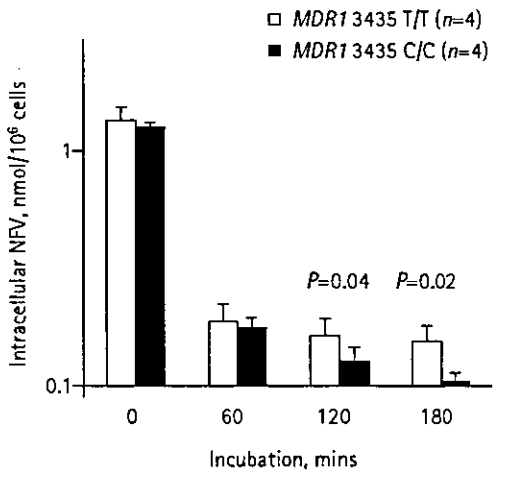
To examine the influence of *MDR1* 3435 genotypes on the response to treatment, we assessed increase in CD4 cell counts and viral suppression in 21 patients after initiation of HAART. At first, we hoped to analyse data obtained from a group of patients receiving NFV alone as a PI, but could not, due to the small number of NFV-receiving patients. Thus, we carried out the analysis in those patients receiving PIs including NFV (n=11), indinavir (n=4) and saquinavir/lopinavir/ritonavir (n=6). CD4 cell counts before treatment were similar among patients with various genotypes. Patients with various genotypes at *MDR1* 3435 showed similar changes in CD4 cell counts (Figure 5A) and viral suppression (Figure 6A) during 9 months of HAART. We found patients with the *MDR1* 1236 T/T showed higher increase in CD4 cell counts at 1 month (148 cells/μl) and 9 months (264 cells/μl) after initiation of therapy than those with *MDR1* 1236 C/C (20 cells/μl and 34 cells/μl, respectively) (Figure 5C). We suspected that *MDR1* 1236 T/T was associated with a higher rate of recovery of CD4 cell counts for patients receiving HAART with PI. We did not find differences in rates of viral suppression among the patients with various *MDR1* 1236 genotypes (Figure 6C). We did not observe a statistical difference in CD4 cell counts or viral loads among patients with different *MDR1* 2677 genotypes (Figures 5B and 6B).

Figure 3. A typical time course of NFV uptake



LCL cells (1 ×10⁶/10 ml) were incubated in medium containing 10 μM of NFV. Cells were harvested at 0, 5, 60, 120 and 180 min and assayed for intracellular NFV by HPLC. The horizontal axis shows the incubation time in min. The vertical axis shows the intracellular amount of NFV per 10⁶ cells. The error bars represent the standard deviations.

Figure 4. NFV efflux from patients' LCLs

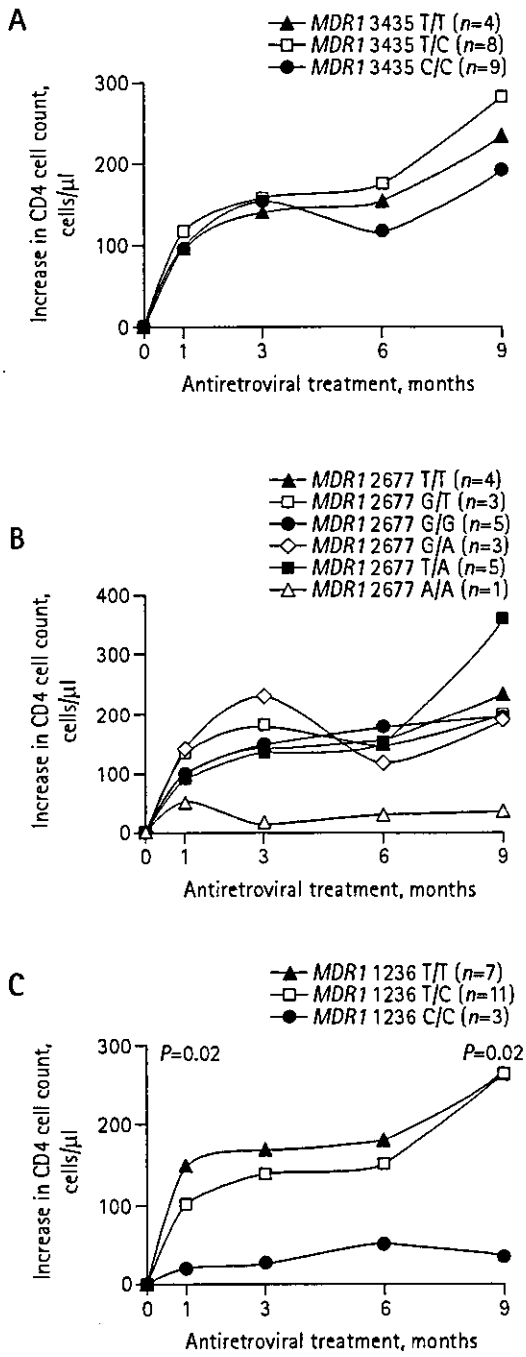


LCL cells were incubated in medium containing 10 μM of NFV for 3 h. Cells were then washed and cultured in NFV-free medium. Intracellular concentration of NFV was determined at 0, 60, 120 and 180 min by HPLC. The horizontal axis shows the incubation time in min. The vertical axis shows the intracellular amount of NFV per 10⁶ cells. We selected eight patients (described in the legend to Figure 2) and examined the velocity of NFV efflux from those cells. The intracellular concentration of NFV was measured several times in all patients' LCLs, and data were similar in every test. The error bars represent the standard deviations.

Discussion

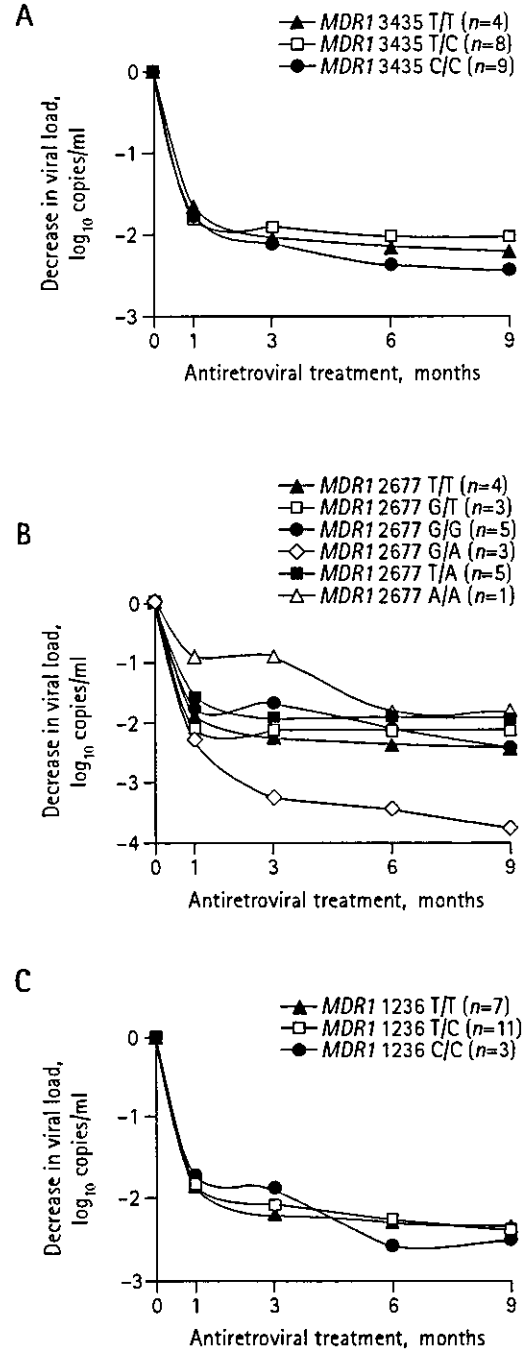
In this study, we genotyped three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) (Figure 1) in 79 HIV-positive Japanese patients and found incomplete linkage disequilibrium – as has also been reported in other ethnic groups [6]. We found that genotype frequencies of the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 3435 (exon 26) in this population were in Hardy–Weinberg equilibrium. This suggested that the studied population was precisely genotyped and unbiased in terms of the *MDR1* gene. We compared the activity of P-gp among patients' LCLs with different *MDR1* 3435 genotypes by measuring NFV efflux from the cultured LCL cells by HPLC. We found that the intracellular concentration of NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with the C/C genotype at 120 min and 180 min. This difference was statistically significant (P=0.04 and 0.02, respectively; Mann–Whitney U-test; Figure 4). In contrast, in the retrospective evaluation of 21 HIV-positive patients

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



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

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



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

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

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

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

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

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