

Frequent Transmission of Cytotoxic-T-Lymphocyte Escape Mutants of Human Immunodeficiency Virus Type 1 in the Highly HLA-A24-Positive Japanese Population

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Although Japan is classified as a country with a low prevalence of human immunodeficiency virus type 1 (HIV-1), domestic sexual transmission has been increasing steadily. Because 70% of the Japanese population expresses HLA-A24 (genotype HLA-A*2402), we wished to assess the effect of the dominant HLA type on the evolution and transmission of HIV-1 among the Japanese population. Twenty-three out of 25 A24-positive Japanese patients had a Y-to-F substitution at the second position [Nef138-10(2F)] in an immunodominant A24-restricted CTL epitope in their HIV-1 *nef* gene (Nef138-10). None of 12 A24-negative Japanese hemophiliacs but 9 out of 16 patients infected through unprotected sexual intercourse had Nef138-10(2F) ($P < 0.01$). Two of two A24-positive but none of six A24-negative Australians had Nef138-10(2F). Nef138-10(2F) peptides bound well to the HLA-A*2402 heavy chain; however, Nef138-10(2F) was expressed poorly on the cell surface from the native protein. Thus, HIV-1 with Nef138-10(2F) appears to be a cytotoxic-T-lymphocyte escape mutant and has been transmitted frequently by sexual contact among the highly A24-positive Japanese population.

While cytotoxic T lymphocytes (CTLs) exert immune pressure on human immunodeficiency virus type 1 (HIV-1) throughout the course of primary and chronic infection (4, 24, 30), HIV-1 escapes through a variety of immune evading mechanisms such as downregulation of HLA class I molecules by Nef (7, 32, 33, 36) and defects in differentiation and maturation of CTLs (2, 6, 27, 35). Viral mutation also plays a crucial role in immune escape, and CTL escape mutant viruses may appear early or late in the clinical course of infection (5, 14, 31). Mutations leading to CTL escape may occur at amino acid residues essential for major histocompatibility complex binding (8), for T-cell-receptor recognition (10), or in flanking regions that affect antigen processing (3, 26).

HIV-1 CTL escape mutants may be stable. One such example at the HLA-B27-restricted Gag epitope, which is related to slower disease progression in adults, could be transmitted vertically from mother to child (12). Although significant association between HLA alleles and polymorphism in reverse transcriptase sequences in a large cohort of patients indicated HIV-1 adaptation at a population level (28), direct horizontal transmission of CTL escape mutants is yet to be shown.

Japan is classified as a country of low HIV-1 prevalence; however, national HIV-1 and AIDS surveillance has shown a steady increase of HIV-1 and AIDS cases mainly through un-

protected sexual intercourse (USI) (84% of HIV-1 patients and 71% of AIDS patients were infected through USI within the country) (1). The Japanese population is less polymorphic than other populations in that 70% express HLA-A24 (genotype HLA-A*2402) (13). We speculated that stable CTL escape mutants from HLA-A24 might be transmitted more frequently in Japan than in other countries where the prevalence of HLA-A24 is much lower. We postulated that Japanese hemophiliacs with HIV-1 infection might be a good comparator group since they were infected directly by contaminated blood products from abroad. We therefore examined an immunodominant CTL epitope in the *nef* gene (Nef138-10) in HLA-A24-positive and -negative hemophiliacs and compared the sequence with sequences from those patients infected through USI (13, 18). We included Caucasian Australians infected through USI as another control of transmission of CTL escape mutants in a country where HLA-A24 is less prevalent (19).

MATERIALS AND METHODS

Patient samples. For sequence analysis, blood specimens were collected in EDTA. Plasmas were separated and preserved at -80°C until use. For enzyme-linked immunospot (ELISPOT) assay, peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood and used on the day of the assay. Patient HLA was typed serologically. In selected patients, HLA genotype was determined after written informed consent was obtained. The study was approved by institutional review boards. All patients serologically typed as A24 positive proved to be positive for HLA-A*2402.

RNA extraction and reverse transcription. Viral RNA was extracted from 140 μl of plasma from patients by using the QIAamp viral RNA Mini kit (QIAGEN) and subjected to reverse transcription according to the manufacturer's protocol with SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) and 5 μM random primers (Takara).

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PCR amplification and sequencing. Fifteen microliters of cDNA (a one-sixth volume of the reverse transcription reaction) was subjected to the first PCR. One-tenth of the first PCR was subjected to the nested PCR. PCR was performed by using Ex-Taq (Takara) with 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final extension for 7 min at 72°C. The primer sets are as follows (all nucleotide positions are in accordance with the HIV-1 SF2 strain). For the *env* V3 region, first PCR primer set 1, primers CBE297P (5'-GGTAGAACAG ATGCATGAGGAT-3') (consensus B *env*, nucleotides [nt] 297 to 318) and E7668 M (5'-TTCTCCAATTGTCCCTCATATCTCCTCTCCA-3') (SF2, nt 7668 to 7636) were used; and for the second PCR primer set 1, primers E6554P (5'-ATCAGTTTATGGGATCAAAGCC-3') (SF2, nt 6554 to 6575) and E7353 M (5'-ACAATTTCTGGGTCCTCCTGAGGA-3') (SF2, nt 7353 to 7328) were used. For the first PCR primer set 2, primers E6984P (5'-ACATGGAAT TAGGCCA-3') (SF2, nt 6984 to 7000) and E7395 M (5'-TTACAGTAGAAA AATCCCC-3') (SF2, nt 7395 to 7375) were used; and for the second PCR primer set 2, primers E7028P (5'-GGCAGTCTAGCAGAAGAAGA-3') (SF2, nt 7028 to 7047) and E7353 M (5'-ACAATTTCTGGGTCCTCCTGAGGA-3') (SF2, nt 7353 to 7328) were used. For the first PCR primer set 3, primers P6951 (5'-GACCATGTACAATGTCAGC-3') (SF2, nt 6951 to 6970) and M7592 (5'-CTCTTGTTAATAGCAGCCCT-3') (SF2, nt 7592 to 7573) were used; and for the second PCR primer set 3, primers E6984P (5'-ACATGGAAT TAGGCCA-3') (SF2, nt 6984 to 7000) and E7353 M (5'-ACAATTTCTGGG TCCCTCCTGAGGA-3') (SF2, nt 7353 to 7328) were used.

For the Nef138-10 epitope, first PCR primer set 1, primers n226p (5'-CTCA GGTACCTTTAAGACCAATG-3') (nt 9028 to 9050) and n650m (5'-GAAAG TCCCCAGCGGAAAGTCCC-3') (nt 9474 to 9452) were used; and for the second PCR primer set 1, primers n296p (5'-GGGACTGGAAGGGCTAATT TGGT-3') (nt 9098 to 9120) and n564m (5'-GAAATGCTAGTTTGCTGTCA AAC-3') (nt 9387 to 9365) were used. For the first PCR primer set 2, primers P8923 (5'-TGGAAAAACATGGAGCAATCA-3') (nt 8923 to 8944) and M9290 (5'-TCCTTCATTGGCCTCTTCTAC-3') (nt 9290 to 9270) were used; and for the second PCR primer set 2, primers P8924 (5'-GGAAAAACATGGAGCAA TCAC-3') (nt 8924 to 8945) and M9288 (5'-CTTCATTGGCCTCTTCTACTC-3') (nt 9288 to 9268) were used. For the first PCR primer set 3, primers P8923 (5'-TGGAAAAACATGGAGCAATCA-3') (nt 8923 to 8944) and n694m (5'-C AGCATCTGAGGGACGCCAC-3') (nt 9525 to 9506) were used; and for the second PCR primer set 3, primers n226p (5'-CTCAGGTACCTTTAAGACCA ATG-3') (nt 9028 to 9050) and n532m (5'-TCTCCGCTCCTCCATCCCA-3') (nt 9345 to 9326) were used.

The PCR products were electrophoresed through agarose gels and purified with a Minielute gel extraction kit (QIAGEN) before sequencing. Purified PCR products were directly sequenced. When sequence ambiguities resulted, DNA fragments were subcloned into the pGEM-T vector (Promega) and sequenced. DNA sequencing was performed by using an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems) on a Perkin-Elmer ABI-377 sequencer.

Cells and media. T2-A24, a kind gift from K. Kuzushima, was cultured in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma) and 0.8 mg of G418 (Invitrogen)/ml (25). We transformed PBMCs from an HLA-A*2402-positive person with human T-cell leukemia virus type 1 (HTLV-1) and established an HLA-A*2402- and CD4-positive-T-cell line (KWN-T4). KWN-T4 was cultured with RPMI 1640 supplemented with 25 U of interleukin-2 (Wako)/ml, 100 U of penicillin/ml, 100 U of streptomycin (Invitrogen)/ml, and 10% heat-inactivated FCS (JRH Bioscience). We also established Nef138-10-specific CTL clones as previously described (22). CTL clones were cultured with RPMI 1640 supplemented with 50 U of interleukin-2/ml, 100 U of penicillin/ml, 100 U of streptomycin/ml, and 10% heat-inactivated FCS.

Peptides. Synthetic peptides Nef138-10 (RYPLTFGWCF), 2F (RFPLTFGW CF), 5C (RYPLCFGWCF), and 2F5C (RFPLCFGWCF) were purchased from Sigma-Genosys. All peptides were more than 95% pure as determined by high-performance liquid chromatography and mass spectroscopy.

Peptide binding assays. Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay as previously described (25). T2-A24 cells were incubated at 26°C for 16 h, and then 2×10^5 cells were incubated with peptides at concentrations from 10^{-4} to 10^{-9} M for 1 h at 4°C. After incubation for 3 h at 37°C, the cells were stained with anti-HLA-A24 monoclonal antibody, A11.1 M (11), and an R-phycoerythrin (RPE)-conjugated F(ab')₂ fragment of anti-mouse immunoglobulin (DAKO). The mean fluorescence intensity was measured by FACScalibur (Becton Dickinson).

ELISPOT assay and functional avidity analysis. Freshly prepared PBMCs (20,000 to 50,000) were added to 96-well multiscreen plates (Millipore) which had been precoated with 100 μ l of 5 μ g of anti-gamma interferon (IFN- γ) monoclonal antibody 1-D1K (Mabtech)/ml at room temperature for 3 h and

blocked with RPMI 1640 medium containing 10% FCS for 1 h. The cells were cultured with synthetic peptide Nef138-10 or its derivatives at concentrations from 10^{-5} to 10^{-11} M for 18 h. After the plates were washed, 100 μ l of 1 μ g of biotinylated anti-IFN- γ monoclonal antibody 7-B6-1 (Mabtech)/ml was added and incubated at room temperature for 90 min. After the plates were washed again, 100 μ l of 1:1,000-diluted streptavidin-alkaline phosphatase conjugate (Mabtech) was added and incubated at room temperature for 60 min. Spots were developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad) and counted with a KS ELISPOT compact (Carl Zeiss). The IFN- γ responses to peptide dilutions were expressed as a percentage of the maximal IFN- γ response seen in each individual assay.

Expression of recombinant Nef protein. Mutations were introduced into *nef* derived from HIV-1 strain SF2 by site-directed mutagenesis based on overlap extension (16). Four proline residues in the Nef proline-rich domain that are important for HLA class I down-regulation were replaced by alanine as described previously (36). The wild type and various *nef* mutants were tagged by His₆ and introduced into a Sendai virus vector (SeV) as previously described (36). For Western blot analysis, KWN-T4 cells were infected with various SeVs at a multiplicity of infection of 10 and lysed 20 h after infection. Western blot analysis was performed according to the standard procedure. Anti-His₆ antibody (QIAGEN) and anti-SeV mouse antiserum were used to detect Nef and SeV proteins, respectively.

⁵¹Cr release assay. Cytotoxicity was measured with a standard ⁵¹Cr release assay as previously described (21). Briefly, KWN-T4 was labeled with 100 μ Ci of Na²⁵¹CrO₄ for 2 h and washed three times with R10. Labeled cells (2×10^5) were added to a 96-well round-bottom microtiter plate with a corresponding amount of peptide. After 1 h of incubation, Nef138-10-specific CTL clones were added and incubated for 4 h. When SeV-infected cells were used as target cells, the cells were infected with SeVs at a multiplicity of infection of 10, 20 h before adding the CTLs.

The supernatants were collected and analyzed with a microbeta counter. Spontaneous ⁵¹Cr release was determined by measuring counts per minute in the supernatant of wells containing only target cells (cpm_{spn}). The maximum release (cpm_{max}) was determined by measuring the release of ⁵¹Cr from target cells in the presence of 2% Triton X-100. Specific lysis was determined as follows: specific lysis = (cpm_{exp} - cpm_{spn}) / (cpm_{max} - cpm_{spn}) \times 100, where cpm_{exp} represents the counts per minute in the supernatant of wells containing target and effector cells.

RESULTS

Sexual transmission of HIV-1 with stereotypic amino acid substitution among the Japanese population. Only patients infected with virus subtyped as B by phylogenetic comparison of envelope sequences were included to avoid potential bias introduced by sequence differences across subtypes (data not shown). We extensively sequenced the Nef138-10 epitope and its flanking region from plasma HIV-1 RNA of 23 Japanese hemophiliacs (11 A24-positive and 12 A24-negative individuals) and 30 Japanese (14 A24-positive and 16 A24-negative individuals) and 8 Caucasian Australians (2 A24-positive and 6 A24-negative individuals) infected through USI (Table 1). Ten out of 11 A24-positive but none of A24-negative Japanese hemophiliacs had a Y-to-F amino acid substitution at the second position [Nef138-10(2F)] (Fig. 1A) ($P < 0.01$), suggesting that HLA-A24 selected for Nef138-10(2F). In the case of patients infected through USI, 13 out of 14 A24-positive and 9 out of 16 A24-negative Japanese patients had Nef138-10(2F) by direct sequencing (Fig. 1B) (data not significant). The frequency of Nef138-10(2F) was significantly higher in Japanese A24-negative patients infected through USI than A24-negative hemophiliacs ($P < 0.01$). Two out of two A24-positive but none of six A24-negative Caucasian Australians had Nef138-10(2F) (Fig. 1C). The frequency of Nef138-10(2F) in A24-negative patients infected through USI was significantly higher for Japanese patients than for Australian patients ($P < 0.05$), suggesting that sexual transmission of the variant was more

TABLE 1. Patient profile^a

Patient ID	Sex	HLA type	No. of CD4 cells/ μ l	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				A24-negative Japanese hemophiliacs			
Patient ID	flanking	CTL epitope	flanking	Patient ID	flanking	CTL epitope	flanking
	WQNYTFGPGI	RYPLTFGWCF	KLVFVPEPKV		WQNYTFGPGI	RYPLTFGWCF	KLVFVPEPKV
A24-J041	-----V	-F-----	-----M	NA24-J037	-----	-----	-----
A24-J033	-----E--T	-F-----Y	-----D--	NA24-J035	-----	-----	-----M--
A24-J031	-H-----T	-F-----	-----	NA24-J031	-----	-----	-----G/E-V/I
A24-J030	-----T	-F--C-----	-----	NA24-J041	-----	-----	-----DE
A24-J034	-----T	-F-----	-----DQ-Q	NA24-J032	-----	-----	-----M--
A24-J038	-----	-----C-----	-----D-D--	NA24-J030	--S-----V	-----C-----	-----
A24-J005	-D/E-----T	-F-----	-----	NA24-J040	-----	-----	-----I
A24-J029	-----V/T	-F-----	-----Q-	NA24-J033	-----	-----	-----L/V-----
A24-J037	--C-----T	-F-----	-----D--	NA24-J029	-H-----	-----	-----D-
A24-J035	-----T	-F-----	-----	NA24-J034	-----	-----	-----V/L-----
A24-J036	--C-----T	-F-----	-----	NA24-J039	-----	-----C-----	-----D-D-
				NA24-J006	-----V	-----C-----	-----D--

B

A24-positive Japanese infected through USI				A24-negative Japanese infected through USI			
Patient ID	flanking	CTL epitope	flanking	Patient ID	flanking	CTL epitope	flanking
	WQNYTFGPGI	RYPLTFGWCF	KLVFVPEPKV		WQNYTFGPGI	RYPLTFGWCF	KLVFVPEPKV
A24-J006	-----V	-F-----	-----E/D--Q-	NA24-J025	-H-----V	-----C-----	-----D-D/AQ-
A24-J007	-----T	-F--C-----	-----A--E-	NA24-J023*	-----T	-Y/W/F-----	-----I
A24-J009	-----T	-F-----	-----	NA24-J021	-----	-----	-----N--Q-
A24-J010	-----T	-F-----	-----QR-	NA24-J018*	-----T	-Y/F--C-----	-----Y 8/10, F 2/10
A24-J012	-----T	-F-----	-----D-	NA24-J017*	-----T	-Y/F-----	-----L--
A24-J013	-----T	-F-----	-----D-DQ-	NA24-J016	-----V	-----	-----L--Q-
A24-J016	-D-----V	-----C-----	-----DQD-	NA24-J015	-----T	-F-----	-----D-DQ-
A24-J017	-D-----T	-F--C-----	-----DQ-	NA24-J012	-H/QS-----T	-----	-----D-DQ-
A24-J018	-----T	-F-----	-----I	NA24-J011	-----T	-F-----	-----NQ-
A24-J023	-----T	-F-----	-----L--GEA	NA24-J010	-----	-----	-----
A24-J021	-----T	-F-----	-----D-DQ-	NA24-J009	-----T	-F-----	-----NQ-
A24-J024	-----T	-F-----	-----D-D-	NA24-J008	-D-----T	-F-----	-----L--Q-
A24-J025	-D-----T	-F-----	-----DQDQ-	NA24-J007	-----T	-F-----	-----NQ-
A24-J026	-----T	-F-----	-----KQ-	NA24-J005	-G/D-----T	-F-----	-----DQDQ-
				NA24-J003	-H-----	-----	-----DQ--
				NA24-J002	-Q/HG-----	-----	-----D-DQ-

C

A24-positive Australian infected through USI				A24-negative Australian infected through USI			
Patient ID	flanking	CTL epitope	flanking	Patient ID	flanking	CTL epitope	flanking
	WQNYTFGPGI	RYPLTFGWCF	KLVFVPEPKV		WQNYTFGPGI	RYPLTFGWCF	KLVFVPEPKV
A24-A001	-----T	-F-----	-----	NA24-A007	-----V	-----	-----
A24-A002	-----T	-F-----	-----M-----	NA24-A005	-----V	-----	-----
				NA24-A013	-----	-----	-----
				NA24-A008	-H-----	-----	-----M-P/Q--
				NA24-A003	-H-----	-----	-----D-D--
				NA24-A006	-----	-----C-----	-----G-

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 slash. 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

Patient ID	Sample Date		nef138-10			cloning
	M/D/Y	WQNYTFGPPI	RYPLTFGWCF	KLVPVEPEKV		
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

Patient ID	Sample Date		nef138-10			cloning
	M/D/Y	WQNYTFGPPI	RYPLTFGWCF	KLVPVEPEKV		
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 rec-

ognized 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).

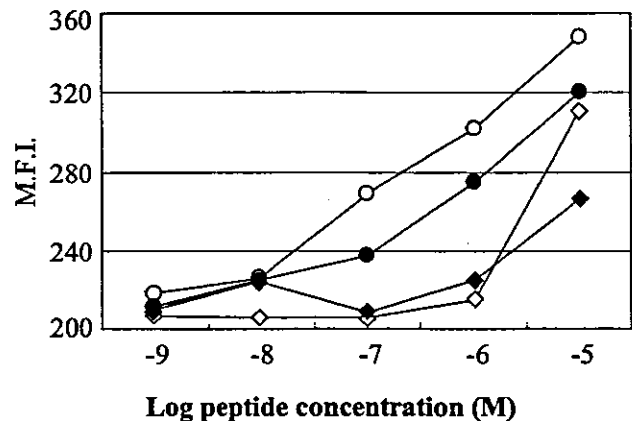


FIG. 3. Binding of the wild-type and mutant peptides to HLA-A*2402 molecules. Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay. Symbols: ○, wild type; ●, 2F; ◇, 5C; ◆, 2F5C. M.F.I., mean fluorescence intensity.

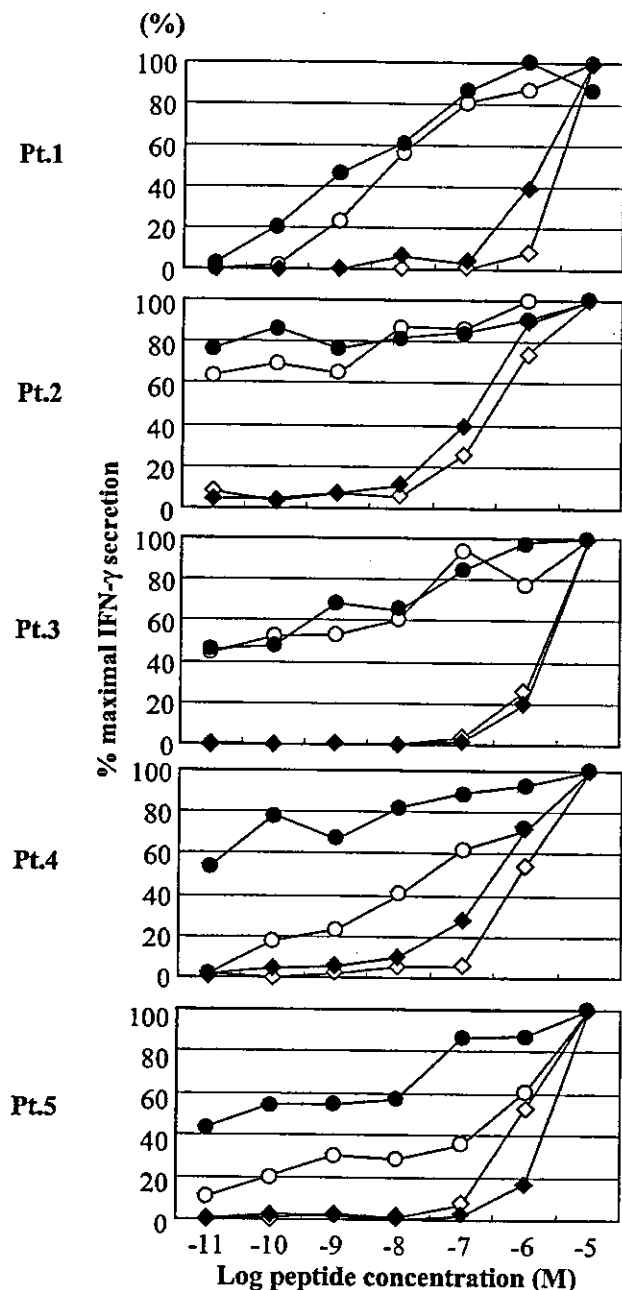


FIG. 4. Functional avidity assay. The reactivity of peptide-specific cells in PBMCs from five patients against log-fold dilutions of peptide was determined. Symbols: ○, wild type; ●, 2F; ◇, 5C; ◆, 2F5C.

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

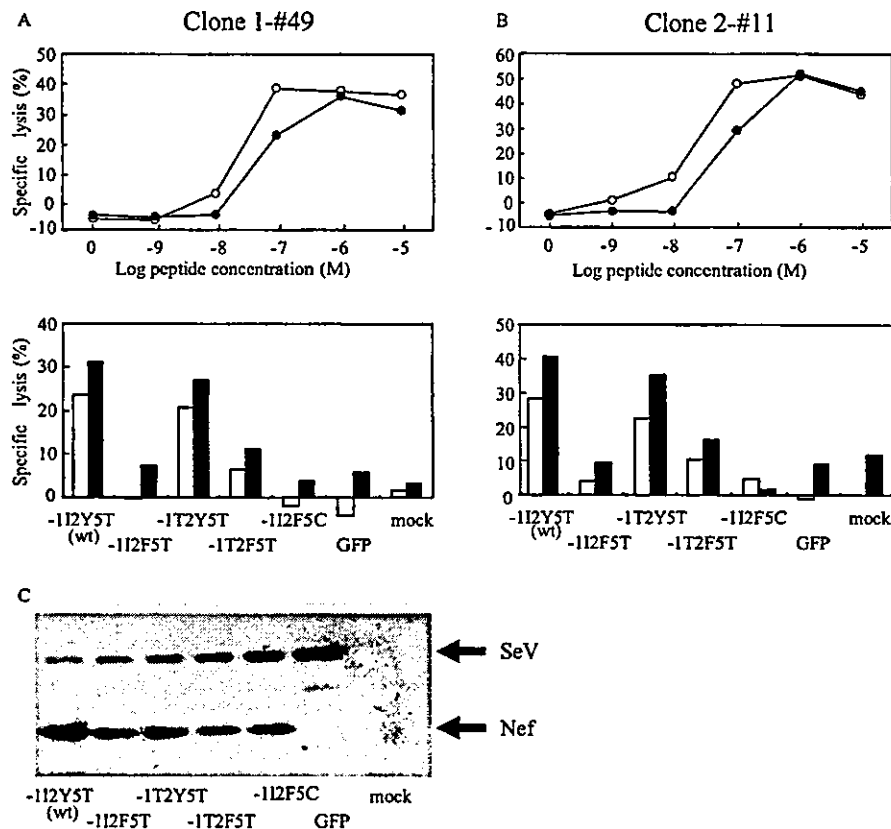


FIG. 5. Killing activity of clone 1-#49 (A) against KWN-T4 target cells pulsed with log-fold dilutions of peptide (top) and expressing native Nef proteins containing wild-type sequences (-1I2Y5T), a Y-to-F substitution at the second position of the CTL epitope (-1I2F5T), an I-to-T substitution at the -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 μ g) of the same KWN-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 ($1 \times 10^6/10$ 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

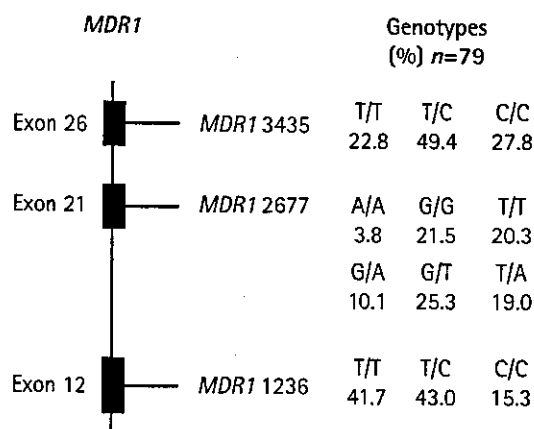
The patients' frozen LCL cells were extracted with 1.5 ml of ethanol. The extracts were then clarified by centrifugation at 2050 ×g for 10 min at 4°C. The ethanol extracts were evaporated at 30°C and dissolved in 180 µl of mobile phase, which was a mixture of phosphate buffer (containing 50 mM KH_2PO_4 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 C₁₈ 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

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*

$$p: \text{Frequency for the T allele } \frac{33 \times 2 + 34}{2 \times 79} = 0.633$$

$$q: \text{Frequency for the C allele } 1 - p = 0.367$$

$$*79 \times p^2 = 31.7$$

$$†79 \times 2pq = 36.7$$

$$*79 \times q^2 = 10.6$$

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*

$$p: \text{Frequency for the T allele } \frac{18 \times 2 + 39}{2 \times 79} = 0.475$$

$$q: \text{Frequency for the C allele } 1 - p = 0.525$$

$$*79 \times p^2 = 17.8$$

$$†79 \times 2pq = 39.4$$

$$*79 \times q^2 = 21.8$$

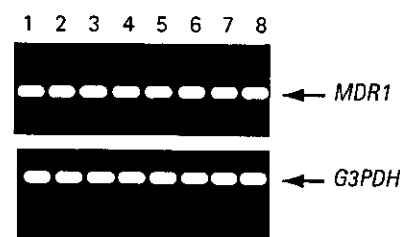
the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21, excluding the genotypes containing G) and *MDR1* 3435 (exon 26), we found significant linkage disequilibrium between *MDR1* 2677 A (T) and *MDR1* 1236 C (T), *MDR1* 2677 A (T) and *MDR1* 3435 C (T), and *MDR1* 1236 C (T) and *MDR1* 3435 C (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

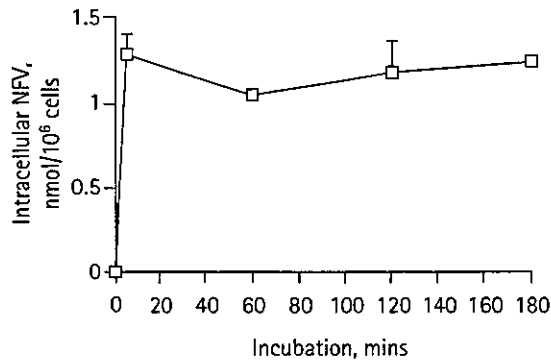
Figure 2. *MDR1* mRNA expression in LCLs



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).

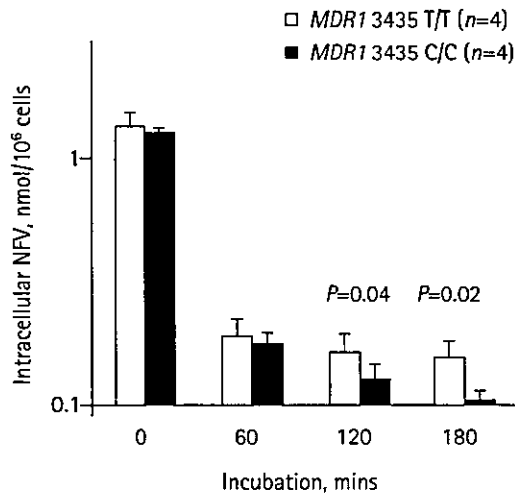
RT-PCR (Figure 2). The concentration of intracellular NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with C/C genotype at 120 min and 180 min. This difference was statistically significant ($P=0.04$ and 0.02 , respectively, Mann-Whitney U-test, Figure 4). This meant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

Figure 3. A typical time course of NFV uptake



LCL cells ($1 \times 10^6/10$ ml) were incubated in medium containing $10 \mu\text{M}$ 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 10^6 cells. The error bars represent the standard deviations.

Figure 4. NFV efflux from patients' LCLs



LCL cells were incubated in medium containing $10 \mu\text{M}$ 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 10^6 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.

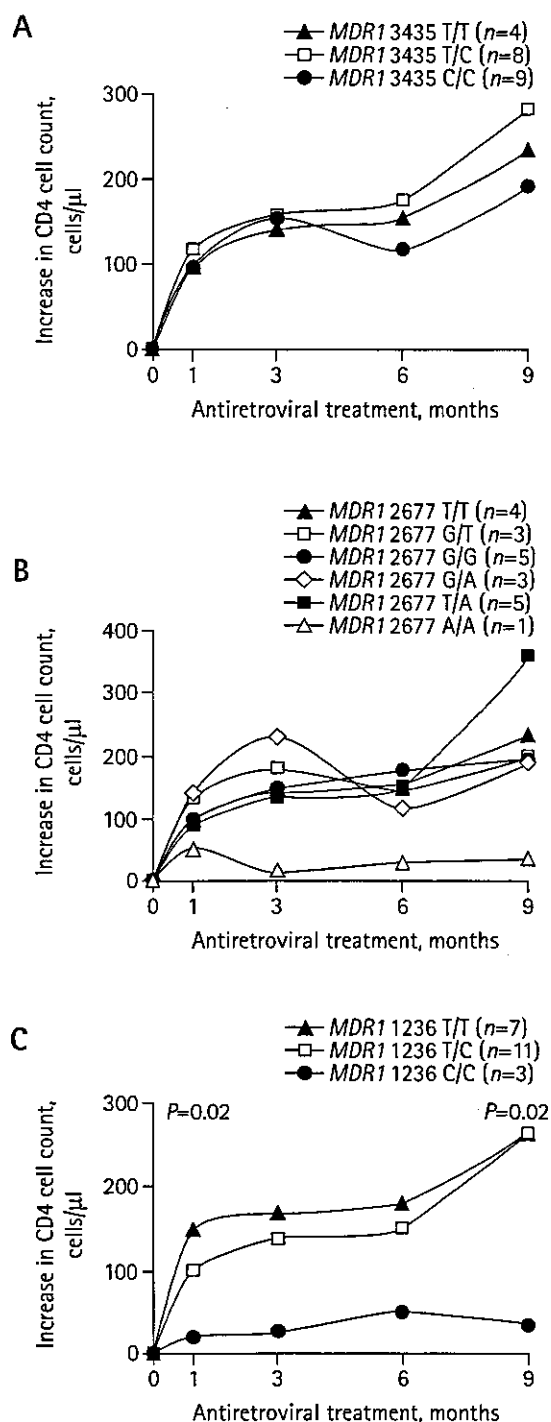
genotype was slower than that with C/C genotype. Thus, we suspect the activity of P-gp in patients' LCLs with the *MDR1* 3435 T/T genotype is lower than that with the C/C genotype.

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).

Discussion

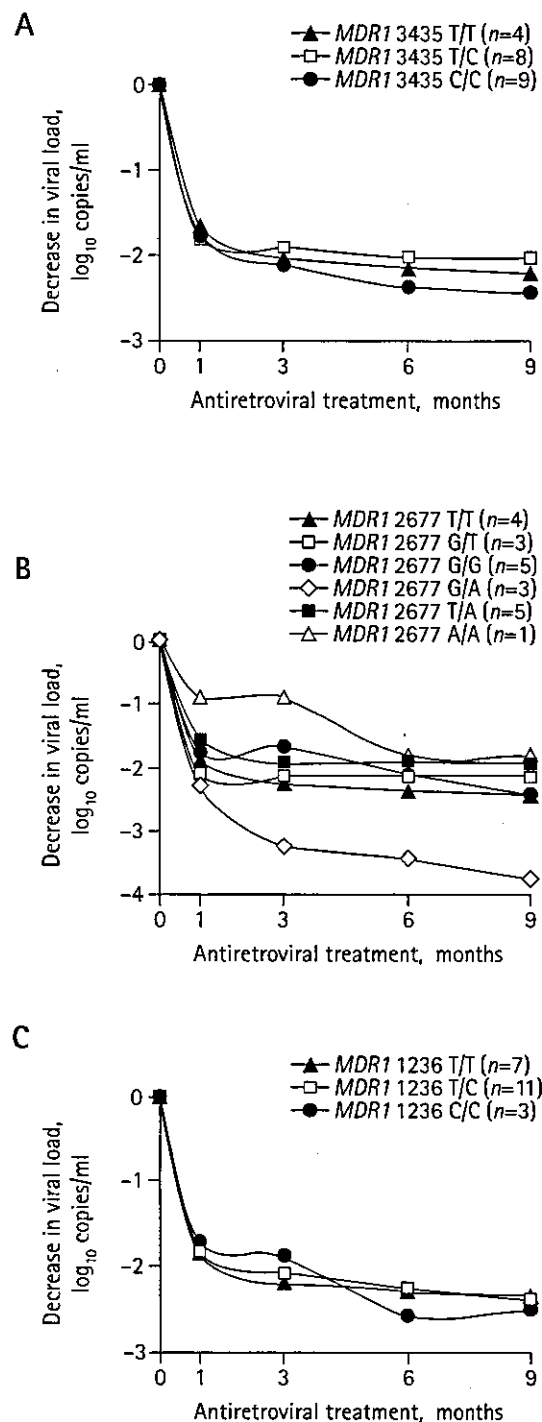
In this study, we genotyped three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) (Figure 1) in 79 HIV-positive Japanese patients and found incomplete linkage disequilibrium – as has also been reported in other ethnic groups [6]. We found that genotype frequencies of the 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 μ M), the uptake of NFV seems active rather than passive. However, these *in vitro* data depart from what has been observed in *in vivo* measurements of NFV in patients [19,20], presumably due to the presence of alpha(1)-acid glycoprotein to which NFV binds in plasma [21]. Furthermore, this discrepancy may also be due to the differential distribution of NFV among tissues rather than in free artificial medium. Therefore, our *in vitro* data should be considered as such, that is, *in vivo* lymphocytes may be unlikely to have this high intracellular to extracellular concentration ratio (250:1).

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

Although an *in vitro* study showed that the velocity of NFV efflux in patients' LCLs with the *MDR1* 3435 T/T genotype was slower than that with the C/C genotype, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 genotypes (Figures 5A and 6A). Four equally possible accounts seem to explain this discrepancy. Firstly, since the C/C genotype at *MDR1* 3435 is also correlated with higher expression of P-gp in intestinal epithelial cells that adsorb PIs, the *MDR1* 3435 C/C is likely to be associated with higher absorption of PIs and higher PI concentration in

plasma [12,22]. The higher plasma levels of NFV in 3435 C/C patients in one study [12] is puzzling and as yet not fully understood. Secondly, the sample size ($n=21$) in this study may be too small to evaluate CD4 cell counts or viral suppression in a statistical way. Thirdly, since the enrolled patients received different treatment combinations of PIs and reverse transcription inhibitors during antiretroviral therapy, the clinical evaluation was not normalized. Finally, because LCLs – immobilized B cells – but not CD4+ T cells were used in this study, the function of P-gp in a setting of HIV-1 infection may not have been accurately tested. In contrast to the *MDR1* 3435, we observed a marginal but statistically significant association of the *MDR1* 1236 SNP with the CD4 cell count increase although this SNP is a silent mutation. To our knowledge, this clinical association of *MDR1* 1236 with statistical significance is unprecedented, although its clinical significance remains to be investigated. In conclusion, a large-scale and case-controlled study would be required to test whether SNPs of *MDR1* affect the clinical course during antiretroviral therapy with PIs and the prognosis of infected patients.

Acknowledgements

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A CCR2-V64I polymorphism affects stability of CCR2A isoform

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Objective: A valine to isoleucine substitution at position 64 of CCR2 (*CCR2-64I*) is associated with a delay in progression to AIDS in HIV-1-infected individuals. The aim of the present study is to elucidate the molecular mechanism underlying the effect of this allele.

Design: We analysed the effect of the 64I substitution on levels of expression of CCR2A and CCR2B, two CCR2 isoforms produced by alternative splicing.

Methods: Sendai virus vector was used to express CCR2 molecules.

Results: While CCR2B trafficked well to the cell surface, CCR2A, which differs from CCR2B only by the sequence of its C-terminal cytoplasmic tail, was detected predominantly in the cytoplasm. The level of expression of CCR2A-64I was significantly higher than that of CCR2A without the substitution. On the other hand, the 64I substitution did not affect levels of CCR2B expression. Pulse-chase experiments revealed that the 64I substitution increased the half-life of CCR2A in cells. When co-expressed with CCR5, CCR2A-64I interfered more severely with cell surface expression of CCR5 than did wild-type CCR2A. Furthermore, immunoprecipitation experiments showed that CCR2A co-precipitated with an immature form of CCR5.

Conclusion: These results suggest that CCR2A binds to CCR5 in the cytoplasm and down-modulates its surface expression. We propose that the increased ability of CCR2A-64I to down-modulate CCR5 expression might be a possible cause of a delay in HIV-1 disease progression in patients with this allele.

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Introduction

The chemokine receptor CCR2B has been regarded as a minor HIV-1 coreceptor because only a small number of HIV-1 strains has been shown to use CCR2B as an entry coreceptor [1–3]. Nevertheless, a polymorphism in the CCR2 gene, *CCR2-64I*, has been reported to be associated with delayed disease progression in HIV-1 infected individuals in several Caucasian cohorts [4–8]. This polymorphism, a G-to-A transition at position 190, changes CCR2B codon

64 from valine to isoleucine, introducing a conservative amino acid change into the first transmembrane domain. It was unclear why a single amino acid substitution in a minor coreceptor could affect HIV-1 disease progression, as there was no difference in HIV-1 co-receptor activity between the variant CCR2B-64I and CCR2B without the 64I substitution (*CCR2B-64V*) [9,10]. Furthermore, these studies also excluded the possibility that CCR2B-64I exerts a dominant-negative effect on the expression and activity of CCR5.

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It is possible that the *CCR2* polymorphism may be linked to other polymorphisms in genes that influence AIDS progression. The *CCR2* gene is located approximately 15 kb from the 5' end of the *CCR5* gene, and the *CCR2-64I* allele is indeed linked to a certain *CCR5* promoter haplotype [11]. However, experiments using promoter-reporter fusion constructs showed that the *CCR5* promoter haplotype, which is in a strong linkage disequilibrium with *CCR2-64I*, did not affect transcriptional activity of the *CCR5* promoter [10]. Thus, the mechanism underlying the protective effect of *CCR2-64I* against AIDS progression still remained to be elucidated.

Two alternatively spliced *CCR2* isoforms, *CCR2A* and *CCR2B*, were reported to be present in freshly isolated human monocyte, THP-1, and MonoMac 6 leukaemia cell lines [12,13]. An open reading frame encoded in the chromosome corresponds to *CCR2B*,

while alternatively spliced transcripts produce *CCR2A*. The two *CCR2* isoforms differ only in their C-terminal cytoplasmic tails (Fig. 1). Therefore, an individual carrying the *CCR2-64I* allele also produces *CCR2A* molecules with isoleucine at position 64. Although the cytoplasmic tail spans less than one-fifth of the entire *CCR2* molecule, this difference caused a drastic alteration in their localization in cells [13]. While *CCR2B* trafficked well to the cell surface, *CCR2A* was detected predominantly in the cytoplasm. A progressive truncation study of the C-terminal cytoplasmic tail indicated that a cytoplasmic retention signal(s) was located in the C-terminal cytoplasmic tail [13]. Nevertheless, *CCR2A* molecules that successfully trafficked to the cell surface could respond to the stimulation of monocyte chemoattractant protein (MCP)-1 in a similar fashion to *CCR2B* [14].

As none of the previous studies investigated the effect

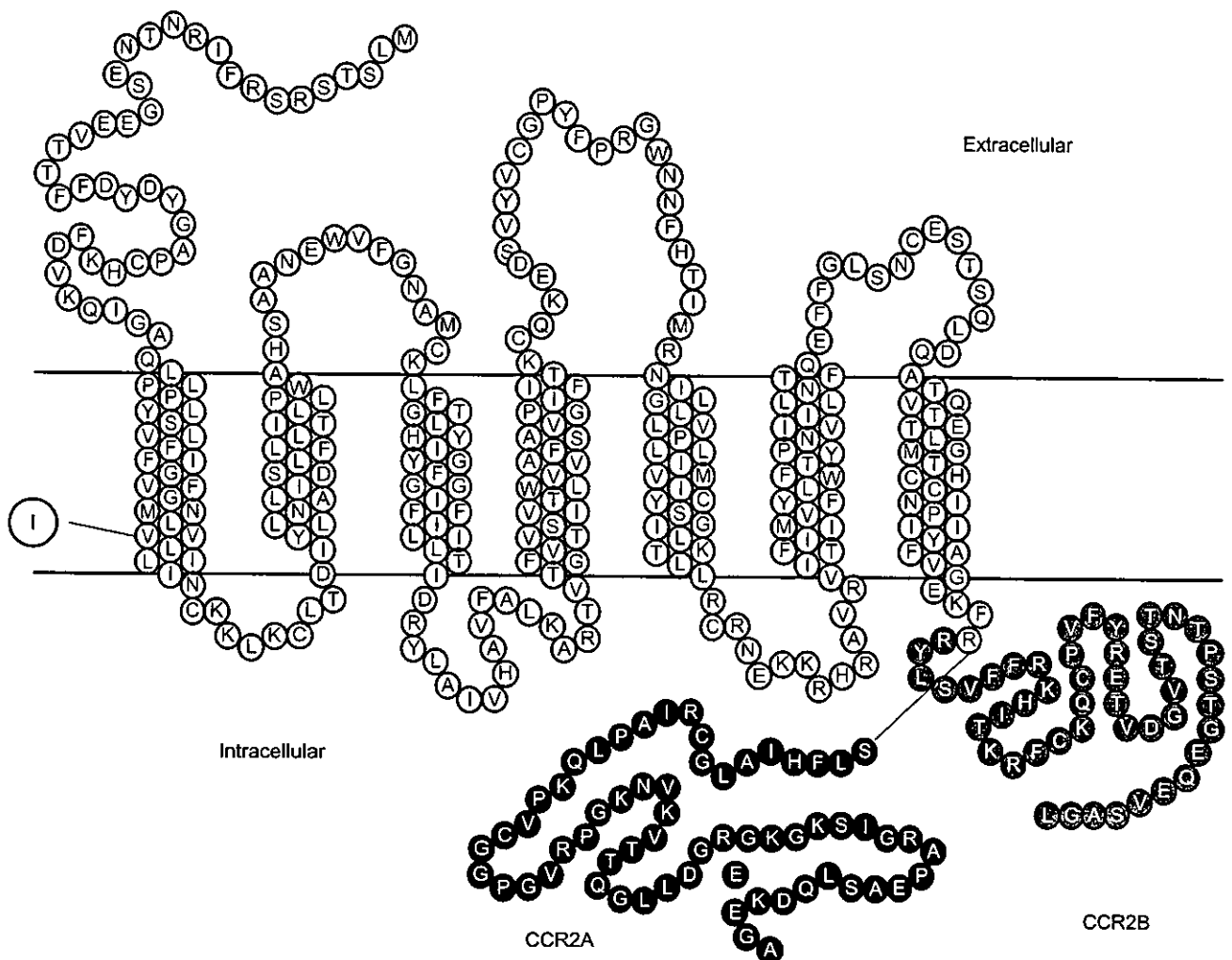


Fig. 1. The structure of the CCR2A and CCR2B molecules. Outlined letters in grey circles denote amino acid residues present in CCR2B. Outlined letters in black circles denote amino acid residues present in CCR2A. A letter I in a large circle denotes a substitution at position 64.

of the 64I substitution on CCR2A molecules, we generated recombinant Sendai viruses (SeV) expressing either CCR2A-64V or CCR2A-64I. Here we show that the 64I substitution indeed affected the stability of CCR2A molecules in cells, and increased the ability of CCR2A to down-modulate the major HIV-1 co-receptor, CCR5.

Materials and methods

Generation of recombinant SeV

THP-1 cells were shown to possess both CCR2-64V and CCR2-64I alleles by using a standard genotyping method [15]. Therefore, CCR2A-64V, CCR2A-64I, CCR2B-64V, and CCR2B-64I cDNA were obtained by reverse transcription (RT)-PCR from mRNA extracted from THP-1 cells and then inserted to the *NotI* site of pSeV18+b(+). The entire coding regions in the resultant plasmids were verified for sequence authenticity as well as for the presence or absence of the 64I substitution. For generating CCR2A-64V and CCR2A-64I cDNA carrying a c-myc-tag (EQKLI SEEDL) at their C-termini, cloned CCR2A-64V and CCR2A-64I cDNA served as templates for PCR amplification using a primer containing a nucleotide sequence corresponding the c-myc-tag fused with the C-terminal portion of CCR2A. Recombinant SeV carrying CCR2A-64V, CCR2A-64I, CCR2B-64V, CCR2B-64I, or C-myc-tagged versions of CCR2A-64V and CCR2A-64I were recovered according to a previously described method [16]. The wild-type Z strain of SeV served as a control in all the experiments.

Generation of a recombinant vaccinia virus

For generating CCR5 cDNA carrying a HA tag (YPYDVPDYAA) at its C terminus, cloned CCR5 cDNA served as a template for PCR amplification by using a primer containing a haemagglutinin (HA) tag sequence fused with the C-terminal portion of CCR5. The resultant PCR products were then inserted into pNZ68K2-Not. The entire coding region of CCR5-HA was verified for sequence authenticity. A recombinant vaccinia virus (Vac) was recovered from the resultant plasmid according to previously described procedures [17].

Flow cytometric analysis

CV1 monkey kidney cells, U937 monocytic cells and Jurkat T cells were infected with recombinant SeV expressing CCR2A-64V, CCR2A-64I, CCR2B-64V, or CCR2B-64I. Five to 18 h after infection, cells were incubated with MAB150, a mouse monoclonal antibody (MAb) against CCR2 (R & D Systems, Minneapolis, Minnesota, USA). Antibodies bound to cells were detected using fluorescein-5-isothiocyanate (FITC)-conjugated goat antibody directed against

mouse IgG (Cappel, Aurora, Ohio, USA). CV1 or H9 cells infected with SeV expressing CCR2A-64V, CCR2A-64I, CCR2B-64V, or CCR2B-64I were superinfected with a recombinant Vac expressing CCR5, CXCR4, or CD4 at 9 h after SeV infection. After incubation for 5 h at 37°C, cells were stained for CCR5 using T227 rat MAb against CCR5 [17] followed by FITC-conjugated goat anti-rat IgG; for CXCR4 using 12G5 mouse MAb (R & D systems) followed by FITC-conjugated goat anti-mouse IgG; or for CD4 using FITC-conjugated anti-human CD4, Leu3a (Becton Dickinson, San Jose, California, USA), and analysed by FACScan (Becton Dickinson).

Immunofluorescence microscopy

CV1 cells expressing CCR2A or CCR2B were fixed and permeabilized before being incubated with MAB150 antibody as described previously [17]. Bound antibodies were then detected using FITC-conjugated goat antibody against mouse IgG. Indirect immunofluorescence was visualized using a Lasersharp2000 Confocal Microscope System (Bio-Rad, Hercules, California, USA). Anti-Calnexin (Stressgen, San Diego, California, USA) or anti-Giantin (CRPinc, Berkeley, California, USA) rabbit polyclonal antibody was used with Cy5-conjugated goat antibody against rabbit IgG (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Chemotaxis assay

Chemotaxis assays were performed according to previously described methods [18]. Briefly, MCP-1 (PeproTech, Rocky Hill, New Jersey, USA) diluted at an indicated concentration of chemotaxis buffer (RPMI 1640 with 0.25% human serum albumin) was added to the bottom chamber of a 5- μ m pore polycarbonate Transwell culture insert (Costar; Corning, New York, USA). Jurkat cells were infected with a SeV expressing CCR2A-64V or CCR2A-64I and incubated at 37°C for 4 h. Cells were then washed with RPMI1640 and re-suspended in chemotaxis buffer and added to the upper chamber of the insert. Transmigrated cells in 4 h at 37°C were counted using a FACScan.

Pulse-chase analyses of CCR2A and CCR5

CV1 or U937 cells were infected with a SeV expressing CCR2A-64V-myc or CCR2A-64I-myc. Nine hours after infection, cells were labelled with 500 kbq/ml of EXPRE³⁵S³⁵S[³⁵S] protein labelling mix (> 37 Tbq/mmol; PerkinElmer (Boston, Massachusetts, USA) in amino acid-free medium for 30 min. For CCR5 analysis, cells were infected with a recombinant Vac expressing CCR5-HA, incubated at 37°C for 5 h and then labelled. Cells were then washed, fed with fresh medium and incubated for 0, 15, 30, 60, or 120 min at 37°C, chilled on ice, and lysed in lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). CCR2A

and CCR5 proteins in the lysates were precipitated with anti-myc mouse MAb (9B11; Cell Signaling, Beverly, Massachusetts, USA) and anti-HA high affinity rat MAb (Roche, Indianapolis, Indiana, USA), respectively, using a Protein G Immunoprecipitation Kit (Roche). Precipitated materials were subjected to SDS-PAGE on a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Groningen, Netherlands), and the amount of radiolabel incorporated was visualized on a BAS Imager (Fujix, Kanagawa, Japan).

Gene reporter fusion assay

A recombinant Vac-based gene activation assay using a β -galactosidase gene as a reporter was performed as described previously [19]. Briefly, mouse fibroblast L cells were transfected with β -galactosidase reporter plasmid pGINT7 β -gal and infected with a recombinant Vac expressing gp160 of an R5 HIV-1 strain SF162. At the same time, CV1 cells were infected with SeV expressing CCR2A-64V or CCR2A-64I and incubated at 37°C for 9 h. Cells were then superinfected with recombinant Vacs expressing T7 RNA polymerase, human CD4, and CCR5, detached by trypsinization, and cultured at 37°C for 5 h. Then, L and CV-1 cells were mixed, incubated for 3 h, and β -galactosidase activities in the cell lysate were measured by using chlorophenol red- β -D-galactopyranoside as substrate.

HIV-1 productive infection

MT4 cells (4×10^5) were infected with SeV expressing CCR2A-64V, CCR2A-64I or parental Z strain of SeV at a multiplicity of infection (MOI) of 40 plaque forming unit (PFU)/cell mixed with SeV expressing CCR5 at an MOI of 10 PFU/cell and incubated at 37°C for 5 h. Cells were then superinfected with 60 ng p24 of an R5 HIV-1 strain SF162. The culture supernatants were collected periodically and p24 levels were measured.

Immunoprecipitation and western blot analysis

CV1 cells were infected with SeV expressing CCR2A-64V-myc or CCR2A-64I-myc, and incubated at 37°C for 9 h. Cells were then superinfected with a Vac expressing CCR5-HA and incubated at 37°C for 5 h and then lysed. CCR2A-64V-myc, CCR2A-64I-myc or CCR5-HA proteins were immunoprecipitated, and subjected to SDS-PAGE as described above. Proteins were then electrophoretically transferred to a PVDF membrane (Immobilon; Millipore, Bedford, Massachusetts, USA). Blots were blocked and probed with the antibodies overnight at 4°C and then incubated with peroxidase-conjugated anti-mouse (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA) or anti-rat IgG (American Qualex, San Clemente, California, USA) and developed using the Immun-Star HRP chemiluminescent kit (Bio-Rad).

Results

Expression of CCR2A and CCR2B

We generated a recombinant SeV expressing either CCR2A-64V or CCR2B-64V. Confocal microscopic observations (Fig. 2a) and flow cytometric analyses (Fig. 2b) confirmed the different subcellular localization of these two CCR2 isoforms. In CCR2B-64V expressing CV1 cells, fluorescent signals of CCR2 were observed mainly on the cell surface. In contrast, CCR2A-64V was localized predominantly to the cytoplasm, although a small portion of CCR2A was observed on the cell surface. In the cytoplasm, signals of an endoplasmic reticulum marker calnexin were only partially co-localized with CCR2A signals (Fig. 2a, left), whereas the majority of signals for the Golgi marker giantin overlapped with those of CCR2A (Fig. 2a, right). These results suggested that most CCR2A molecules were retained in the Golgi.

To assess the effect of the 64I substitution on CCR2A expression, we generated a recombinant SeV expressing CCR2A-64I and compared levels of expression of CCR2A-64I with those of CCR2A-64V. As shown in Fig. 2b, CCR2A-64I showed slightly but significantly higher levels of expression than CCR2A-64V in various cell types, despite the same promoter being used. The mean fluorescence intensity (MFI) of CCR2A-64I and CCR2A-64V was 274 and 140 in CV1, 133 and 40 in U937 monocystic cells, and 29 and 21 in Jurkat T cells. The difference was greater in U937 cells than in Jurkat cells. The difference was also observed at 5, 12, and 18 h after infection of recombinant SeVs (Fig. 2c). Exactly the same result was obtained when recombinant SeV expressing C-myc-tagged versions of CCR2A-64V (CCR2A-64V-myc) and CCR2A-64I (CCR2A-64I-myc) were used (Fig. 2c). In contrast, we failed to detect any difference in the levels of expression between CCR2B-64V and CCR2B-64I (MFI 2698 and 2663, respectively; Fig. 2b), as had been described in the previous reports [9,10]. Northern blot analyses confirmed that there was no difference in the amount of CCR2 mRNA among cells expressing CCR2A-64V, CCR2A-64I, CCR2A-64V-myc, CCR2A-64I-myc, CCR2B-64V and CCR2B-64I (data not shown). These data clearly indicate that the substitution of valine to isoleucine affects levels of cell surface expression of CCR2A, but not of CCR2B.

Chemokine receptor activity of recombinant CCR2A-64V and CCR2A-64I

To determine whether or not CCR2A molecules expressed by a recombinant SeV fully retained chemokine receptor activity, we performed a chemotaxis assay. As shown in Fig. 2d, both cells expressing CCR2A-64V and CCR2A-64I migrate toward MCP-1. However, cells expressing CCR2A-64I migrated