Virus infection

At 24 and 48 h post-transfection, the resultant virion was purified and used for infection into M8166/H1luc cells (Nagao et al., 2004), which contain integrated reporter DNA carrying HIV-1 long terminal repeat (LTR) and luciferase. Upon infection with HIV-1, HIV-1 LTR is activated along with the expression of viral transactivator Tat, and luciferase expression in cytoplasm is induced. For our study, the cells (1×10^6) were infected at 37 °C with 300 μl of three serially diluted viruses. After 90 min, the cells were washed with PBS, added to 1.5 ml of media and cultured for 40 h at 37 °C. The infected cells were then washed with PBS and lysed with 125 μl of Glo lysis buffer (Promega), and a 50 μl sample of each lysate was assayed for photon emission after the addition of 50 μl of Bright-Glo Reagent (Promega) with a microplate luminometer (Centro LB 960; Berthold Technologies, Bad Wildbad, Germany).

Western blotting analysis

The lysates from the pelleted virus particles and cell lysates were prepared as described previously (Willey et al., 1988), while the proteins were resolved on SDS–4–12% polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes. ECL western blotting detection reagents (Nakalai Tesque, Kyoto, Japan) were used to detect VSV-G or HIV-1 antigen on the membranes. Briefly, the membranes were incubated for 1 h at room temperature with anti-VSV-G polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA), the serum of HIV-1 infected patients, or anti-HIV-1 CA-p24 monoclonal antibody (Advanced Biotechnologies Inc., Columbia, MD) and washed. They were then incubated for another 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-human IgG (Vector Laboratories, Burlingame, CA), washed, and visualized by chemiluminescence with LAS-1000 (Fujifilm, Tokyo, Japan) according to the manufacturer's manual. The relative intensity of each band was quantified by digital image analysis using ImageGauge software (Fujifilm).

Cell cytometry analysis

The 293T cells were harvested, washed twice with PBS (−), and fixed by suspending in 1% formaldehyde–PBS(−). The fixed cells were analyzed by FACScan (Bekton Dickinson, Franklin Lakes, NJ) to measure the expression of GFP.

Genomic DNA preparation

Transfected cells were harvested at 12, 24, 48, and 72 h post-transfection, washed twice with PBS(−), and pelleted. GenElute mammalian genomic DNA miniprep kit (Sigma, St. Louis, MO) was used to extract total DNA of the cells. DNA was digested overnight with *Dpn*I restriction enzyme at 37 °C to eliminate contaminating plasmids which were methylated.

Alu PCR analysis

For integrated proviral DNA quantitation, a modified Alu-PCR method from a recent report (Brussel and Sonigo, 2003) was employed. In the first round of PCR, two outward-facing Alu primers that anneal within the conserved regions of the Alu repeat element were used together with an HIV-1 LTR specific primer (L-M667) to optimize the probability of amplifying an LTR sequence since Alu elements could be present in either orientation relative to the integrated provirus. L-M667 consisted of an HIV-1 LTR-specific sequence fused with a lambda phage-specific tag sequence at the 5′ end (Brussel and Sonigo, 2003). For the second round of PCR (real-time PCR), a lambda-specific primer (Lambda T) (Brussel and Sonigo, 2003) was used as a sense primer to detect only the amplified fragments in the first round of PCR, and a Taqman probe and an anti-sense primer were selected from the set for R/U5 DNA detection in the previous report (Julias et al., 2001). Cycling conditions of the first round of PCR were 94 °C for 3 min followed by 22 cycles of 94 °C for 30 s, 66 °C for 30 s, 70 °C for 10 min, and then 72 °C for 10 min (Ikeda et al., 2004). Equal volume of DNA (0.15 μg) was applied for amplification. Ten-fold serially diluted DNA samples of 72 h post-transfection with VSV-G pseudotyping were employed as standards. Total amount of DNA in each standard was adjusted by adding total DNA of mock-transfected cells. The resultant PCR products were diluted 100-fold and subjected to real-time PCR using ABI7500 (Applied Biosystems, Foster City, CA).

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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 unprotected 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′-TTCTCCAATTGTCCCTCATATCTCCTCCTCCA-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′-ACAATTTCTGGGTCCCCTCCTGAGGA-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 AATTCCCC-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′-ACAATTTCTGGGTCCCCTCCTGAGGA-3′) (SF2, nt 7353 to 7328) were used. For the first PCR primer set 3, primers P6951 (5′-GACCATGTACAAATGTCAGC-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′-ACATGGAA TTAGGCCA-3′) (SF2, nt 6984 to 7000) and E7353 M (5′-ACAATTTCTGGG TCCCCTCCTGAGGA-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′-CTTCATTGGCCTCTTCTACCT-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′-TCTCCGCGTCCTCCATCCCA-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 μl of 5 μg 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 μl of 1 μg 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 μl 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 His_6 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-His_6 antibody (QIAGEN) and anti-SeV mouse antiserum were used to detect Nef and SeV proteins, respectively.

^{51}Cr release assay. Cytotoxicity was measured with a standard ^{51}Cr release assay as previously described (21). Briefly, KWN-T4 was labeled with 100 μCi of $Na_2^{51}CrO_4$ for 2 h and washed three times with R10. Labeled cells (2×10^3) 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 ^{51}Cr 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 ^{51}Cr 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 phylogenic 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/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[bd]	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[bd]	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[bd]	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[bd]	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[bd]	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-----	-----D----

B

A24-positive Japanese infected through USI

Patient ID	flanking WQNYTPGPGI	CTL epitope RYPLTFGWCF	flanking KLVPVEPEKV
A24-J006	----------V	-F--------	-----E/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

Patient ID	Sample Date M/D/Y	nef138-10 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

Patient ID	Sample Date M/D/Y	nef138-10 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).

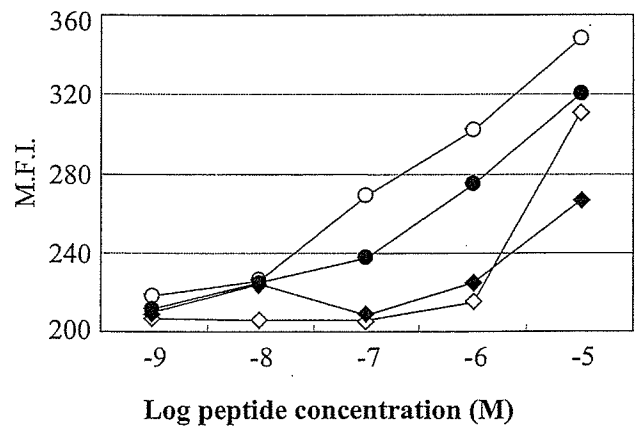

FIG. 3. Binding of the wild-type and mutant peptides to HLA*2402 molecules. Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay. Symbols: ○, wild type; ●, 2F; ◇, 5C; ◆, 2F5C. M.F.I., mean fluorescence intensity.

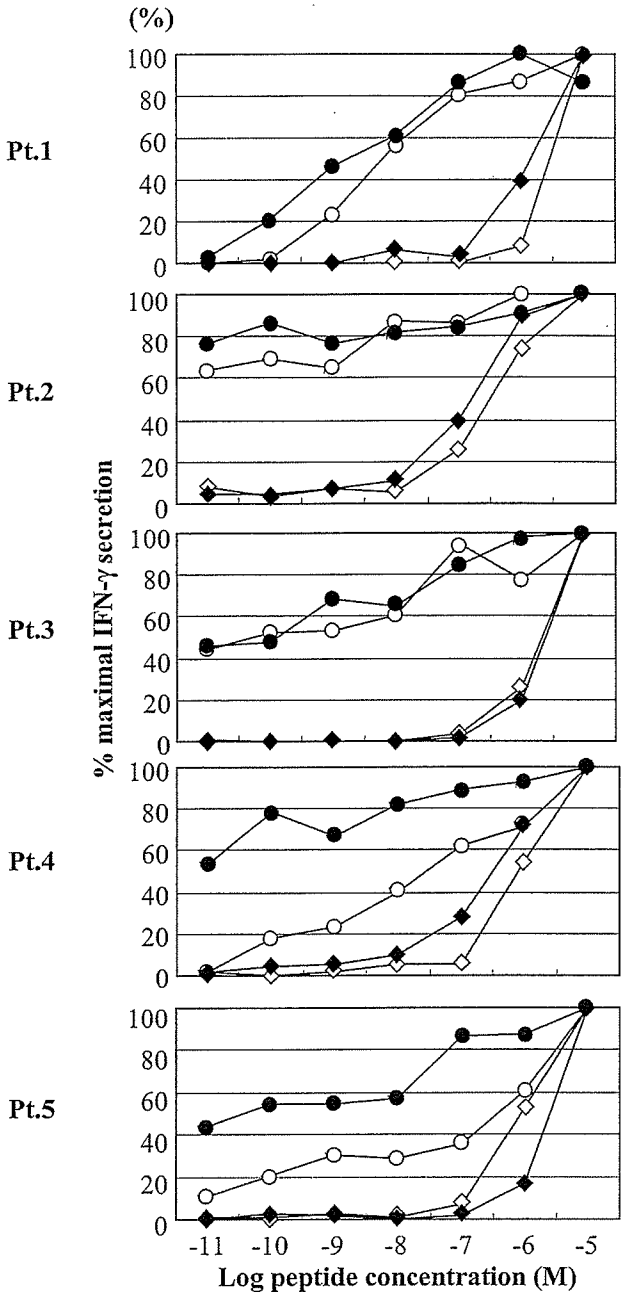

FIG. 4. Functional avidity assay. The reactivity of peptide-specific cells in PBMCs from five patients against log-fold dilutions of peptide was determined. Symbols: ○, wild type. ●, 2F; ◇, 5C; ◆, 2F5C.

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

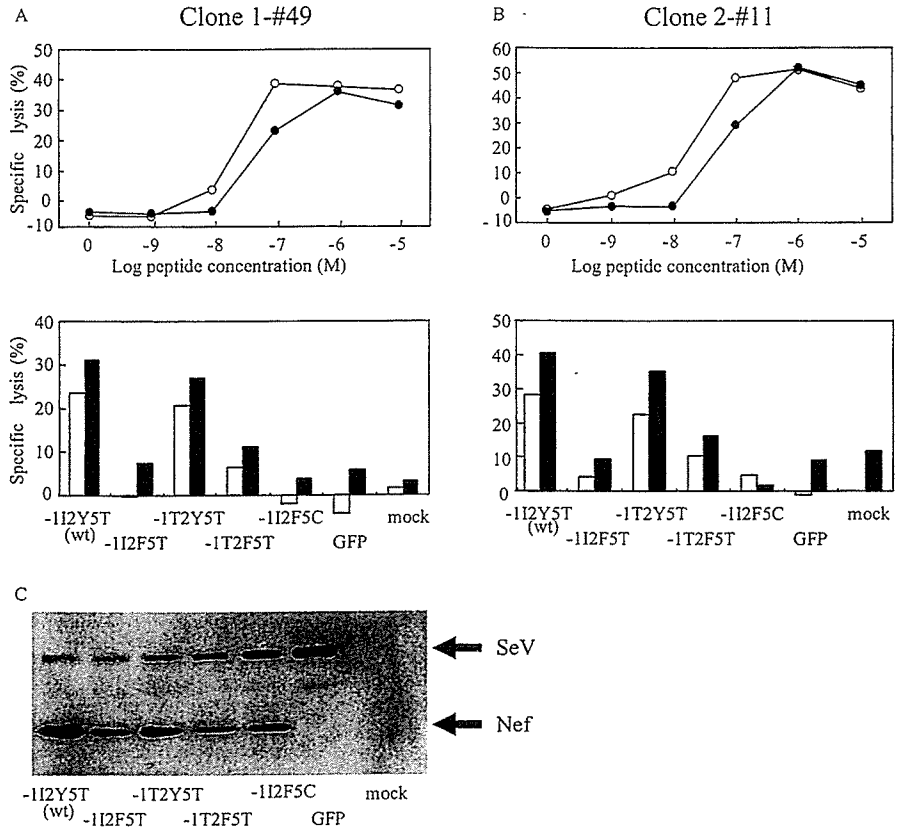

FIG. 5. Killing activity of clone 1-#49 (A) against KWN-T4 target cells pulsed with log-fold dilutions of peptide (top) and expressing native Nef proteins containing wild-type sequences (−1I2Y5T), a Y-to-F substitution at the second position of the CTL epitope (−1I2F5T), an I-to-T substitution at the −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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Frequent Detection of Epstein-Barr Virus and Cytomegalovirus but Not JC Virus DNA in Cerebrospinal Fluid Samples from Human Immunodeficiency Virus-Infected Patients in Northern Thailand

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Applying nested-PCRs, we frequently detected DNA of Epstein-Barr virus and cytomegalovirus but not JC virus in cerebrospinal fluid samples from 140 human immunodeficiency virus-infected patients with central nervous system symptoms in northern Thailand. Despite the low incidence of primary central nervous system lymphoma or cytomegalovirus encephalitis among Thai AIDS patients, Epstein-Barr virus and cytomegalovirus infections in the central nervous system are common.

According to reports from the Thai Ministry of Public Health, opportunistic infections are common in the central nervous system (CNS) of Thai AIDS patients and have caused a significant portion of mortality. Cryptococcal meningitis was noted as 20.3% of the first AIDS defining illness in northern Thailand; toxoplasma encephalitis was 5.3%; tuberculous meningitis was also seen, though the exact prevalence in the total number of AIDS patients is unknown (2). Virus infections in the CNS such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), and JC virus (JCV) can result in life-threatening consequences as they cause primary CNS lymphoma, cytomegalovirus encephalitis, and progressive multifocal leukoencephalopathy, respectively. In developed countries, PCR tests to detect EBV, CMV and JCV DNA in the cerebrospinal fluid (CSF) have been used as a supplemental diagnostic test (5). However, in developing countries, such a test is not available and very limited data have been reported about the prevalence of virus infections in the CNS. The objective of this study is to investigate the significance of EBV, CMV, and JCV infections in the CNS of human immunodeficiency virus (HIV)-infected Thais in northern Thailand.

From March 2001 to June 2003, CSF samples of 140 HIV-1-infected patients at the day care center clinic or the HIV/AIDS ward in Lampang Hospital, which is a Thai government referral hospital for Lampang province in northern Thailand, were examined as they were clinically suspected of having opportunistic infections in the CNS and did not have any contraindication for lumbar puncture. Consequently, 163 CSF samples including follow-up CSF samples were taken. All CSF samples were initially examined for routine laboratory tests such as cell count, protein concentration, sugar level, bacterial and fungal culture, Indian ink stain, Gram stain, acid-fast bacilli stain, and a latex agglutination test for cryptococcal antigen (PASTOREX, Bio-Rad, France). After the routine laboratory tests, residual CSF samples were stored at −80°C until DNA extraction.

All study patients gave informed consent when they participated in the Lampang HIV cohort study, which was approved by the Thai government ethics committee. DNA was extracted from 200 μl of CSF (QIAGEN blood mini DNA extraction kit, QIAGEN, California), eluted with 50 μl of distilled water, and 10 ul were used as the target for PCR. PCR amplifications were performed using ExTaq DNA polymerase (TaKaRa Biomedical, Osaka, Japan) and nested primer sets targeting specific sequences of virus genes as previously published: the EBNA-1 gene for EBV (PCR product, 209 bp) (3), immediately early protein gene for CMV (146 bp) (1), and regulatory regions for JCV (approximately 396 bp) (7, 10).

Diagnosis of EBV and CMV infection was made on the basis of the size of amplicons, but for the diagnosis of JCV, we further sequenced PCR products. The positive control for EBV PCR was DNA extracted from Namalwa cells as previously described (11). DNA extract from culture supernatant of CMV-producing fibroblast cells was used as a positive control for CMV PCR. DNA extract from the urine of a healthy JCV carrier was used as a positive control for JCV PCR. The detection limit of nested PCR for EBV and CJV was evaluated as previously described (10, 11). The detection limit of CMV PCR was approximately 100 copies/ml of CSF, which was estimated by a limiting dilution method using a DNA sample, of which the number of CMV copies was determined by a quantitative real-time PCR (Mitsubishi-Kagaku BCL, Tokyo, Japan).

The median (interquartile range; range) of age among 140 patients was 33 years (30 to 37 years; 20 to 63 years); 93 patients (66.4%) were male. $CD4^+$ T-cell count data were

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TABLE 1. Clinical characteristics of study patients[a]

Parameter	No. of patients (%)
Symptoms	
Altered mental status	37 (27.0)
Focal sign	18 (13.1)
Chronic headache	121 (88.3)
Fever	104 (75.9)
Diagnosis of CNS infection	
Cryptococcal meningitis[b]	98 (70.0)
Toxoplasmic encephalitis[b]	10 (7.1)
Tuberculous meningitis	6 (4.3)
Aseptic meningitis	3 (2.1)
No diagnosis	24 (17.1)
Antiretroviral drug therapy	
None	131 (93.6)
Two drugs	3 (2.1)
Three drugs	4 (2.9)
Unknown	2 (1.4)
Status at discharge	
Improved	99 (70.7)
Dead	29 (20.7)
Referred to another hospital	7 (5.0)
Unknown	5 (3.6)

[a] Medical records were available for 137 patients.
[b] Includes one case with both cryptococcal meningitis and toxoplasmic encephalitis.

available in 48 patients; the median (IQR; range) was 16 (7 to 42/μl; 0 to 605/μl). Clinical pictures of the patients are summarized in Table 1. Cryptococcal meningitis was by far the most common opportunistic infection in the CNS. There was no case of primary CNS lymphoma, CMV encephalitis, or progressive multifocal leukoencephalopathy. However, one patient developed clinical symptoms of progressive multifocal leukoencephalopathy during the follow-up period.

Thirty-one of 140 patients (22.1%) were positive for EBV PCR and 16 of 140 (11.4%) patients were positive for CMV PCR. Six patients were positive for both EBV and CMV PCR. More than one CSF sample was collected from 20 patients. The results of CMV PCR were concordant in all pairs of samples, but the results of EBV PCR were discordant in five pairs. None of the 140 first CSF samples was positive for JCV PCR. However, JCV was detected in the second CSF sample of one cryptococcal meningitis case. We found that patients with EBV DNA in the CSF tended to be older than the other patients and had a significantly higher protein concentration and a higher number of cells in the CSF (Table 2). We did not find any factor significantly associated with CMV DNA detection in CSF.

We found that EBV infection in the CNS is common in advanced HIV-infected patients in northern Thailand. This frequency was higher than the result of a similar study in Italy (4). The majority of our study patients were suffering from cryptococcal meningitis, but the detection rate of EBV DNA did not significantly differ according to the clinical diagnosis of cryptococcal meningitis. A significant association of EBV detection with a CSF cell count raised the concern that we may have detected EBV in the lymphocytes circulating in the peripheral blood, which invaded the CSF, rather than EBV of the CNS involvement. However, EBV was also often detected in patients without a CSF cell: EBV DNA was detected in CSF from 9 (19.6%) of 46 patients with a CSF cell count of 0.

Several studies from Western countries have shown a high sensitivity and specificity of EBV PCR in CSF for diagnosing primary CNS lymphoma (5). However, we have not seen any primary CNS lymphoma cases in our experience of having seen over 2,400 HIV-1-infected patients at the day care center clinic from its establishment on October 1995 to July 2004. Furthermore, the government report of adult AIDS patients from 1994 to 1998 showed that there were 98 primary CNS lymphoma cases, which represented only 0.1% of all reported first AIDS-defining illness in Thailand (2). According to the Thai national

TABLE 2. Factors associated with EBV or CMV DNA detection in the CSF[a]

Parameter	Median no. of patients (IQR)					
	EBV			CMV		
	PCR positive (n = 31)	PCR negative (n = 109)	P	PCR positive (n = 16)	PCR negative (n = 124)	P
Age (yr)	35 (31–42)	33 (30–36)	0.069	32 (29–38)	33 (30–37)	0.7
No. female	11 (35.5%)	36 (33.1%)	0.79	6 (37.5%)	41 (33.1%)	0.72
CSF cell count (/μl)	8 (0–66)	4 (0–10)	0.045	6 (0–18)	4 (0–16)	0.97
CSF protein concn (mg/dl)	80 (55–160)	53 (34–90)	0.003	75 (30–88)	57.5 (40–100)	0.96
Clinical diagnosis[b]						
Cryptococcal meningitis	21 (70.0%)	76 (69.7%)		12 (75.0%)	85 (69.1%)	
Toxoplasmisc encephalitis	4 (13.3%)	5 (4.6%)	0.40	1 (6.3%)	8 (6.5%)	0.65
Tubercular meningitis	1 (3.3%)	5 (4.6%)		0 (0.0%)	6 (4.9%)	
Aseptic meningitis	0 (0.0%)	3 (2.8)		1 (6.3%)	2 (1.6%)	
No apparent CNS infection	4 (13.3%)	20 (18.3%)		2 (12.5%)	22 (17.9%)	
Symptoms[c]						
Altered mental status	12 (40.0%)	25 (23.4%)	0.07	4 (25.0%)	33 (27.3%)	0.85
Headache	27 (90.0%)	94 (87.9%)	0.75	15 (93.8%)	106 (87.6%)	0.47
Focal sign	4 (13.3%)	14 (13.1%)	0.97	1 (6.3%)	17 (14.0%)	0.34
Fever	22 (73.3%)	82 (76.6%)	0.71	11 (68.8%)	93 (76.9%)	0.48
Death at discharge[d]	5 (17.2%)	24 (24.2%)	0.43	4 (25.0%)	25 (22.3%)	0.81

[a] Data are median (interquartile range) or number of patients (%).
[b] One case with cryptococcal meningitis and toxoplasmic encephalitis was excluded from the analysis.
[c] Medical records were available for 137 patients.
[d] Survival status at discharge was known for 128 patients.

guideline for clinical management of HIV/AIDS patients (8), if patients with a focal sign have poor response to the toxoplasma encephalitis therapy, further investigation with computed tomography scan is recommended to exclude other space-occupying lesions such as primary CNS lymphoma, and the computed tomography scan is available at most government referral hospitals in Thailand. However, this clinical practice may underdiagnose a minimal primary CNS lymphoma, which does not cause CNS symptoms.

Because of a high mortality rate of symptomatic Thai patients (9), patients with a small primary CNS lymphoma might have died due to other opportunistic infections before the primary CNS lymphoma lesion became large and caused CNS symptoms. Recently the Thai government pharmaceutical organization has started mass production of generic antiretroviral drugs. If many insidious primary CNS lymphoma cases exist in Thailand, we expect to see more patients with apparent primary CNS lymphoma lesions as the antiretroviral drug-treated patients survive longer. Alternatively, it is plausible that Thai patients are less susceptible to the development of primary CNS lymphoma and that EBV DNA detection in CSF from AIDS patients does not supplement the diagnosis of primary CNS lymphoma in Thailand.

In our experiences at Lampang Hospital, CMV retinitis is common among our advanced HIV-infected patients, but we have not seen any case with CMV encephalitis. This rarity of CMV encephalitis may be due to the difficulty of making a firm diagnosis in Thailand, since it requires magnetic resonance imaging or biopsy, which is not widely available, and the disease does not induce characteristic clinical symptoms. Our data on CMV PCR warn that we may be overlooking patients with CMV encephalitis.

Progressive multifocal leukoencephalopathy cases have been reported but are not common in Thailand (2, 6). At Lampang Hospital, we had one male patient who presented with hemiparesis and was diagnosed with progressive multifocal leukoencephalopathy on the basis of computed tomography scan findings and clinical course. His CSF was negative for JCV PCR, but this result does not exclude progressive multifocal leukoencephalopathy as the sensitivity of JCV PCR is not high (5). We found one case in which JCV virus was detected in the CSF of the second lumbar puncture. This patient did not have any other CNS symptoms besides headache, but he died shortly after the diagnosis of cryptococcal meningitis. We think that a low prevalence of JCV DNA detection is compatible with our clinical impression, that is, progressive multifocal leukoencephalopathy cases are there but not common, though more patients would be detected if brain magnetic resonance imaging were available.

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Unrelated cord blood transplantation for a human immunodeficiency virus-1-seropositive patient with acute lymphoblastic leukemia

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The concurrent use of highly active antiretroviral therapy (HAART) improves results of high-dose chemotherapy with autologous stem cell transplantation (SCT) for human immunodeficiency virus-1 (HIV)-associated lymphomas.[1] Recently, successful allogeneic SCT from HLA-matched sibling donors was reported in HIV-infected patients.[2–4] Here, we describe the first case of an HIV-infected patient with acute lymphoblastic leukemia (ALL) who underwent umbilical cord blood transplantation (CBT).

In July 1996, a 23-year-old Japanese woman presented with fever and genital herpes. She was confirmed as seropositive for HIV, probably transmitted from her boyfriend. In March 2001, a real-time quantitative polymerase chain reaction (PCR) analysis showed that the HIV-RNA level was elevated to 25 000 copies/ml (lower limit of detection, 50). The CD4 count decreased to 28/μl. Therefore, HAART consisting of 60 mg stavudine, 300 mg lamivudine, and 600 mg efavirenz was initiated. In July 2001, the HIV-RNA level decreased to 220 copies/ml, and the CD4 count increased to 129/μl. In May 2003, her complete blood count tests showed a white blood cell count (WBC) of 3990/μl with 29% lymphoblasts. Bone marrow (BM) examination showed hypercellularity with 96% lymphoblasts, which were positive for CD4, CD10, CD13, CD19, CD33, CD34, and HLA-DR. Cytogenetic analysis disclosed the presence of t(9;22)(q34;q11) in 12 of 20 metaphases. The $p190^{BCR\text{-}ABL}$ transcript was shown by a reverse transcriptase (RT)-PCR analysis. She was diagnosed as Philadelphia chromosome-positive ALL. She achieved hematological complete remission after two courses of chemotherapy. She has been taking HAART during and after the chemotherapy and her HIV-RNA level continued to be below detectable levels. She was negative for hepatitis B virus surface antigen and anti-hepatitis C virus antibody, and positive for anti-cytomegalovirus antibody. As she had no HLA-matched related or unrelated BM donors, the patient underwent CBT from an unrelated donor with mismatches at two loci (HLA-B and DR) in September 2003 (Figure 1). The numbers of total nucleated cells and CD34-positive cells in the cord

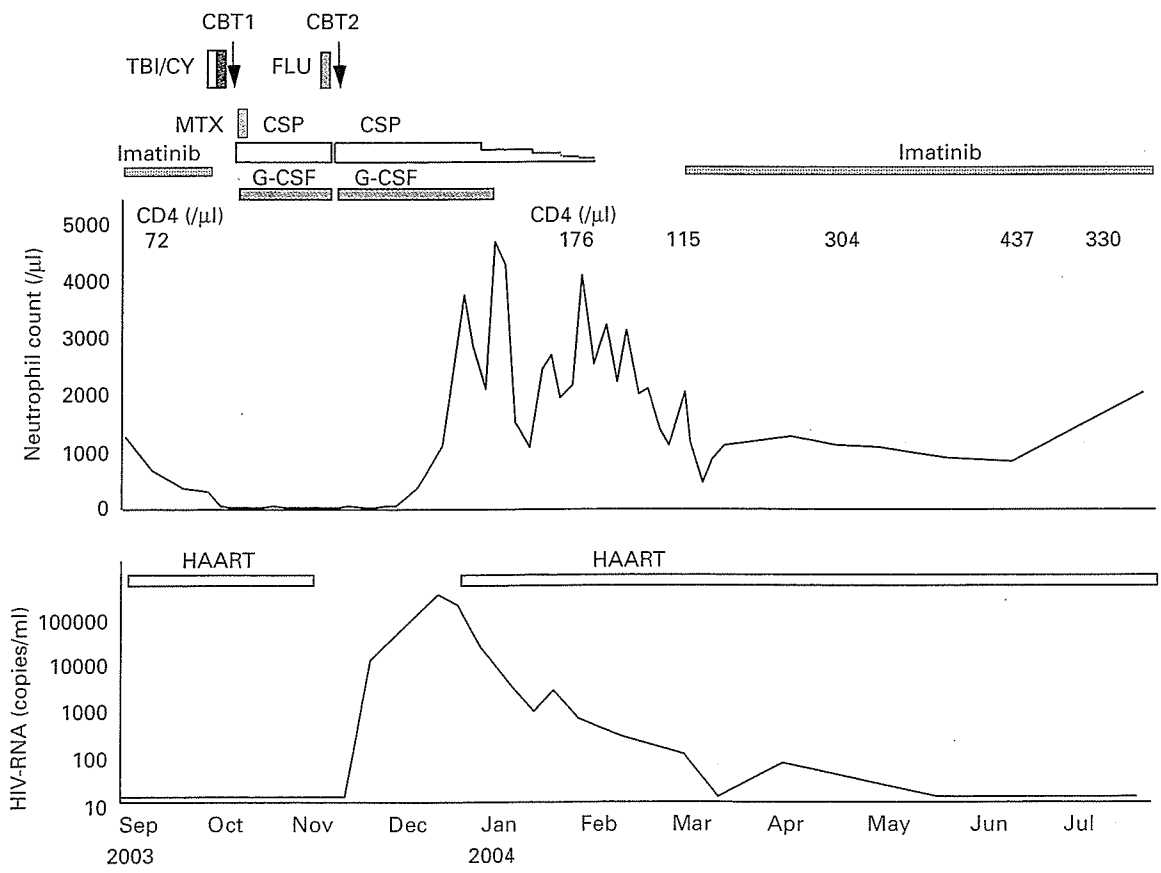

Figure 1 Clinical course of the patient.

blood (CB) unit were 2.9×10^7/kg and 0.76×10^5/kg, respectively. The conditioning regimen included 12 Gy total body irradiation and 120 mg/kg cyclophosphamide. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate. The patient tolerated the procedure well with minimal regimen-related toxicity. Owing to possible myelosuppression, HAART was discontinued on day +28. On day +33, her WBC remained below 100/μl and all of the BM cells were shown to be derived from the recipient. At 40 days after the first CBT, second CBT was performed from an unrelated donor with a one-locus mismatch at HLA-DR. The numbers of total nucleated cells and CD34-positive cells in the CB unit were 2.1×10^7/kg and 0.46×10^5/kg, respectively. The conditioning regimen included 40 mg/m^2 fludarabine for 3 days. Cyclosporine was administered for GVHD prophylaxis. A neutrophil count consistently greater than 500/μl was achieved on day +27. Full donor chimerism of BM cells was shown on day +28. The HIV-RNA level increased to 3×10^6 copies/ml on day +31. After the administration of HAART from day +38, the HIV-RNA levels returned to below detectable levels from day +195, and the CD4 count increased to above 300/μl from day +170. No bacterial or fungal infections were documented during the first and second CBT processes and cytomegalovirus reactivation was successfully treated with ganciclovir and foscarnet. Grade I acute GVHD occurred, but resolved without any additional immunosuppressants. No chronic GVHD was observed. An RT-PCR analysis showed continuous negative test results for the p190$^{BCR\text{-}ABL}$ transcript until the last follow-up evaluation at 15 months post-CBT.

CBT for adults has been associated with a high rate of early transplantation-related mortality (TRM).[5,6] However, our single-institution experience showed a 1-year TRM of 9% and 2-year disease-free survival of 74% in 68 adults after CBT.[7] Both CB donors and the patient in the present study were Japanese. The lesser genetic diversity in a single ethnic population in our studies might be associated with the favorable outcomes of CBT, such as the lower rates of severe acute GVHD. Although our results suggest that CBT is feasible for HIV-infected patients on HAART, the safety and efficacy should be further examined by prospective studies.

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The pro-apoptotic human BH3-only peptide harakiri is expressed in cryptococcus-infected perivascular macrophages in HIV-1 encephalitis patients

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Abstract

In the central nervous system (CNS), HIV-1 targets mainly microglia/macrophages. Like the CD4+ T cell depletion and neuronal loss in AIDS, apoptosis is thought to be involved in eliminating infected macrophages. In this study, we examined the expression of the pro-apoptotic BH3-peptide harakiri (Hrk) in brain tissues of AIDS patients. Immunoreactivity against Hrk was positive in perivascular macrophages infiltrated into some restricted lesions. Most of these immunopositive cells contained small inclusions positive for Grocott's methenamine silver staining. Confocal laser microscopy demonstrated that Hrk expression coincided with immunoreactivities against HIV-1 and *Cryptococcus neoformans*. Expression of Hrk mRNA was demonstrated in these cells by in situ hybridization, which indicated that Hrk is not phagocytosed material. Some pro-apoptotic bcl-family members, including Hrk, may contribute to the delayed hypersensitive reaction in AIDS, in macrophages eliminating opportunistic infection.

Keywords: Apoptosis; Delayed hypersensitivity; *C. neoformans*; Hrk; HIVE

Central nervous system (CNS) involvement in HIV-1 infection is represented by the "AIDS dementia complex", which primarily affects subcortical white matter [13,12]. However, HIV-1 infection is restricted to capillary endothelium, macrophages, and multinucleated giant cells (MGC) in most cases, while astrocytes and neurons are infected only in severe cases [20]. The parenchymal cells (neurons and astrocytes) are probably spared in exchange for the infected perivascular macrophages. Like CD4+ T cell depletion and cortical neuronal loss in AIDS, apoptosis may play an important role in the elimination of infected macrophages. In fact, cell death was demonstrated in HIV-1 infected cultured microglia [19], and some TUNEL-positive macrophages were observed in HIV encephalitis (HIVE) samples [15]. However, this mechanism is not fully understood.

Bcl-2 is an anti-apoptotic peptide cloned from human lymphoma with a chromosomal translocation, t(14;18) [18]. Bcl-2 family members share some of the four homologous domains with bcl-2 (bcl-2 homology regions 1-4; BH1-4). Of these, the BH3 domain is closely linked to pro-apoptotic activity, and the BH3-only proteins Bik/Nbk, Bim, Bod, BLK, and Bnip3 have pro-apoptotic functions.

The bcl-2 family was also demonstrated in the macrophages in HIVE samples. Krajewski et al. reported the expression of Bax, a pro-apoptotic bcl-family peptide, in the microglia/macrophages in HIVE samples [10]. This expression was

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Table 1
Clinical data of autopsied AIDS patients

Patient no.	Age, sex	*C. neoformans*	Additional infection
1	36, M	–	CMV retinitis
2	20, M	–	Toxoplasma meningitis
3	36, M	–	CMV pneumonia
4	37, F	–	Lung toxoplasmosis
5	32, M	–	Lung tuberculosis
6	66, M	–	Glioblastoma
7	69, F	–	PML
8	50, M	–	Lung toxoplasmosis
9	42, M	+	MAI pneumonia
10	30, M	+	PC pneumonia
11	49, M	+	PC pneumonia, CMV retinitis
12	30, M	+	Anal herpes
13	38, M	+	–
14	38, M	+	–
15	34, F	+	–
16	37, M	+	CMV ventriculitis
17	30, M	+	CMV retinitis
18	32, F	+	PC pneumonia, CMV ventriculitis, PML
19	54, M	+	–

CMV: cytomegalovirus MAI: *Mycobacterium avium-intracellulare*; PC: *Pneumocystis carinii*; PML: progressive multifocal leukoencephalopathy.

prominent in the perivascular macrophages, especially in the basal ganglia.

The human protein harakiri (Hrk) and its murine ortholog DP5 belong to this pro-apoptotic BH3-only protein group [8,7]. Both have the cell-death promoting function and heterodimerize with Bcl-2 or Bcl-XL in vitro [8,7]. The expression of Hrk has been demonstrated in lymphoid tissue, pancreas, kidney, liver, lung, and brain. DP5 was originally isolated from an in vitro apoptosis model of the sympathetic neuron [7], and it is expressed in the developing nervous system [9]. Considering these expression patterns, Hrk and DP5 are possibly involved in CNS pathologies. In fact, the upregulation of Hrk and its heterodimer formation with bcl-2 were demonstrated in the anterior horn cells of amyotrophic lateral sclerosis patients [17].

In this study, we examined the expression of Hrk, and discussed its possible contribution to the pathophysiology of HIVE.

The brains of six successive AIDS patients collected at the Institute of Medical Science of the University of Tokyo between 1992 and 1996 were studied. After the preliminary study, we added 13 patients with AIDS (seven patients with opportunistic *Cryptococcus neoformans* (*C. neoformans*) infection were included) from *the Brain Bank of Pitié-Salpêtriére Hospital*, who died between 1991 and 2001. The clinical diagnoses were confirmed by the clinical courses and hematological and virological or bacteriological investigations. The clinical information of the patients is summarized in Table 1. As control cases, we examined 15 patients (Table 2). Controls 1–3 died from systemic disorders and showed no remarkable CNS lesion. Controls 4–11 suffered from cerebral infarction and Nos. 12–15 from inflammatory CNS disorders. Unfortunately, there was no case of *C. neoformans* infection without AIDS in the databases of either institute. All samples were examined for the pathological diagnosis and neuroscience research with the consent of the patient's family.

Table 2
Control cases

Control no.	Age	Sex	Clinical diagnosis
1	28	F	Anorexia nervosa
2	19	M	Duchenne muscular dystrophy
3	76	M	Esophageal carcinoma
4	55	M	Cerebral infarction
5	61	M	Cerebral infarction
6	64	F	Cerebral infarction
7	66	F	Cerebral infarction
8	69	F	Cerebral infarction
9	73	M	Cerebral infarction
10	80	M	Cerebral infarction
11	83	F	Cerebral infarction
12	21	M	Chronic encephalitis
13	43	M	Neuro-Behçet disease
14	35	M	Multiple sclerosis
15	35	F	Multiple sclerosis

Formalin-fixed paraffin-embedded blocks were cut into 3 μm-thick sections for the standard staining methods (hematoxylin and eosin (H&E), Klüver-Barrera, and Grocott), immunohistochemistry, and in situ hybridization. Antisera against human Hrk was raised in immunized rabbits as described elsewhere [7,17]. Other commercial antibodies are summarized in Table 3.

For the immunohistochemistry of HIV core protein p24, deparaffinized samples underwent 15 min of microwave irradiation in citrate buffer (BioGenex, HK086-9K) for antigen retrieval and then were incubated with 0.01% protease type XXIV (Sigma, P8038) for 15 min.

All samples were incubated in 3% hydrogen peroxide in methanol to block endogenous peroxide activity. After incubation in 10% normal bovine serum for 30 min, primary antibody diluted in PBS were was applied for 14 h at 4 °C. After incubation with biotinylated secondary antibodies for 1 h at room temperature, immunoreactivity was made visible using the ABC system (Vector, PK6100) with diaminobenzidine tetrahydrochloride as the substrate.

To investigate the co-localization of Hrk or *C. neoformans* and other antigens (HLA-DR, HIV p24, CMV), we visualized

Table 3
Commercial antibodies

		Clone	Dilution
Mouse monoclonal antisera			
Anti-HIV p24	DAKO	Kal-1	1:50
Anti-cytomagalovirus	MONOSAN	BM204	1:50
Anti-*P. carinii*	DAKO	3F6	1:50
Anti-*Toxoplasma gondii*	NeoMarkers	RB-282-A	1:100
Anti-human HLA-DR	DAKO	CR3/43	1:100
		Lot no.	Dilution
Rabbit polyclonal antisera			
Anti-*C. neoformans*	DAKO	E0123	1:100

the immunoreactivity using laser confocal microscopy. Because the monoclonal antibody against *C. neoformans* (NeoMarkers, CSFi) did not react with our paraffin-embedded samples, we could not confirm the co-localization of *C. neoformans* with Hrk or other rabbit-derived antibodies. For double labeling, sections were incubated with anti-Hrk and another antibody diluted in TBS for 14 h at 4 °C. FITC-conjugated anti-rabbit immunoglobulin was used to visualize the immunoreactivity of Hrk, and Cy3-conjugated anti-mouse immunoglobulin to visualize the second immunoreactivity. Photographs were taken using a Leica TCS 4D system.

In situ hybridization using digoxigenin-labeled probes (sense and antisense) of human Hrk cDNA was described elsewhere [17]. cRNA probes were labeled by the alkaline phosphatase-conjugated anti-digoxigenin antibody, and color was developed with NBT and X-phosphate solutions.

To quantitatively check the local expression patterns of Hrk and its relationship with *C. neoformans* infection, we counted the Hrk-positive macrophages in six cases with *C. neoformans* infection. We randomly selected five vessels with Hrk-positive cells in each case for this purpose. Counting was done under a light microscope instead of a confocal laser microscope, because macrophages are easily identified as foamy cells with continuous cellular membrane. And in the case of counting the *C. neoformans*-positive cells, it was technically difficult to demonstrate the co-localization of two rabbit antibodies against *C. neoformans* and Hrk in single sections. *C. neoformans* positive cells almost always showed granular materials in their cytoplasm and negative macrophages did not contain such substances. We therefore, counted foamy cells with granular materials as *C. neoformans*-positive macrophages.

We first examined the expression of Hrk protein in six Japanese AIDS patients by immunohistochemistry. No Hrk-immunoreactivity was observed in the control cases (cerebral infarction, multiple sclerosis, and encephalitis), even in infiltrated foamy macrophages (data not shown). In contrast, three samples (Nos. 9, 10, 11) from HIVE patients showed strong reactivity against Hrk. Most of the cells exhibiting Hrk immunoreactivity were accumulated around the blood vessels (Fig. 1a). In the brain parenchyma, little immunoreactivity was

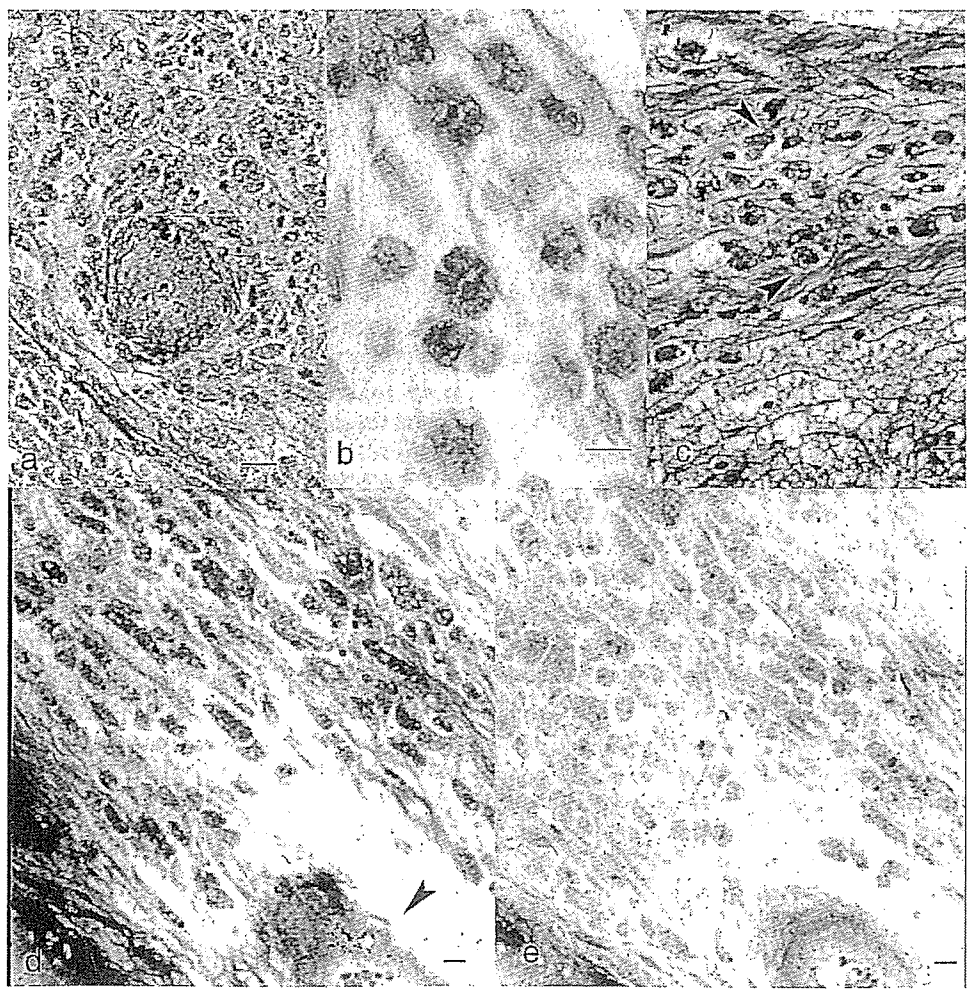

Fig. 1. Immunohistochemistry of Hrk in the basal ganglia of the AIDS patients. (patient 11): (a) immunoreactivity against Hrk was strong in the infiltrated cells around the vessels (bar = 40 μm); (b) these Hrk positive cells had foamy cytoplasm (bar = 10 μm); (c) H&E staining of the same sample. Hrk positive cells were large sized and have foamy cytoplasm. Many cells have small cytoplasmic inclusions (arrow heads) (bar = 20 μm) Hrk mRNA expression in the basal ganglia of the AIDS patients; (d) in situ hybridization of Hrk. Signal was strong in the foamy macrophages around the vessels (arrowhead) (bar = 10 μm); and (e) sense probe (bar = 10 μm).

detected in various other cell types. Morphologically, many of the Hrk-positive cells were relatively large with foamy cytoplasm (Fig. 1b), and some of them were multi-nucleated. Most of the cells contained many small cytoplasmic inclusions that were stained clearly with H&E (Fig. 1c).

We examined the Hrk mRNA expression by in situ hybridization. Consistent with the immunohistochemical analysis, the Hrk mRNA signal was positive in the foamy large perivascular cells of the cases positive for the protein expression (Fig. 1d and e).

These Hrk expressing cells were accumulated around blood vessels and contained some inclusions within the cytoplasm. It should be noted that the Hrk-positive cells were not always detected in perivascular spaces but rather were restricted to the area around only several vessels. They often contained inclusions, and this finding prompted us to test whether an immune response of macrophages against opportunistic infection is involved in Hrk induction. The multiple cytoplasmic inclusions were stained darkly by Grocott technique (Fig. 2a). When viewed using confocal laser scan microscopy, these cells contained immunoreactivity against *C. neoformans* and were positive for HLA-DR antigen (Fig. 2b). These findings indicated that Hrk immunoreactivity was positive in the perivascular macrophages that phagocytose *C. neoformans* in the HIV-1-infected CNS (Fig. 2c), but in other lesions, the expression was faint and scant even in cryptococcus-laden macrophages. This result was also consistent with the clinical information about complicating opportunistic infections. Granular immunoreactivity against HIV p24 was also confirmed, especially in the large foamy cells (Fig. 2d).

However, not all the HIV-positive macrophages were doubly positive for Hrk. We then counted the numbers of total macrophages, those with Hrk immunoreactivity, and those with C. neoformans in autopsied AIDS patients with cryptococcus infection (Fig. 2e). Hrk-positive macrophages almost always contained cryptococcus inclusions, but some cryptococcus-positive macrophages were devoid of Hrk immunoreactivity (Fig. 2e). Close relationship between Hrk expression and opportunistic crypotococcal infection was also implied by the fact that Hrk was virtually negative in the HIV cases without *C. neoformans* infection (data not shown). The proportion of Hrk-positive macrophages in cryptococcus-positive ones varied between vessels and patients (Fig. 2e), suggesting that cryptococcus infection may not directly regulate Hrk expression. In immunocompromised hosts, opportunistic infections of *Toxoplasma gondii*, Cytomegalovirus, *P. carinii*, and *C. neoformans* are frequent, but we did not confirm the common coincident infections of toxoplasma, *P. carinii*, or CMV by immunohistochemistry (data not shown). Some unknown factor(s), in addition to opportunistic infection, may influence Hrk expression in macrophages.

The presence of the Hrk immunoreactivity indicated that: (1) Hrk protein was synthesized in the infected macrophages or (2) the macrophages phagocytosed Hrk-positive cells (e.g., neurons). To test these possibilities, we examined Hrk mRNA expression by in situ hybridization. Consistent with the former hypothesis, the Hrk mRNA signal was positive in the perivascular macrophages of the cases positive for the protein expression. Again, although many macrophages were scattered throughout the brain parenchyma, Hrk mRNA expression was restricted to some perivascular lesions.

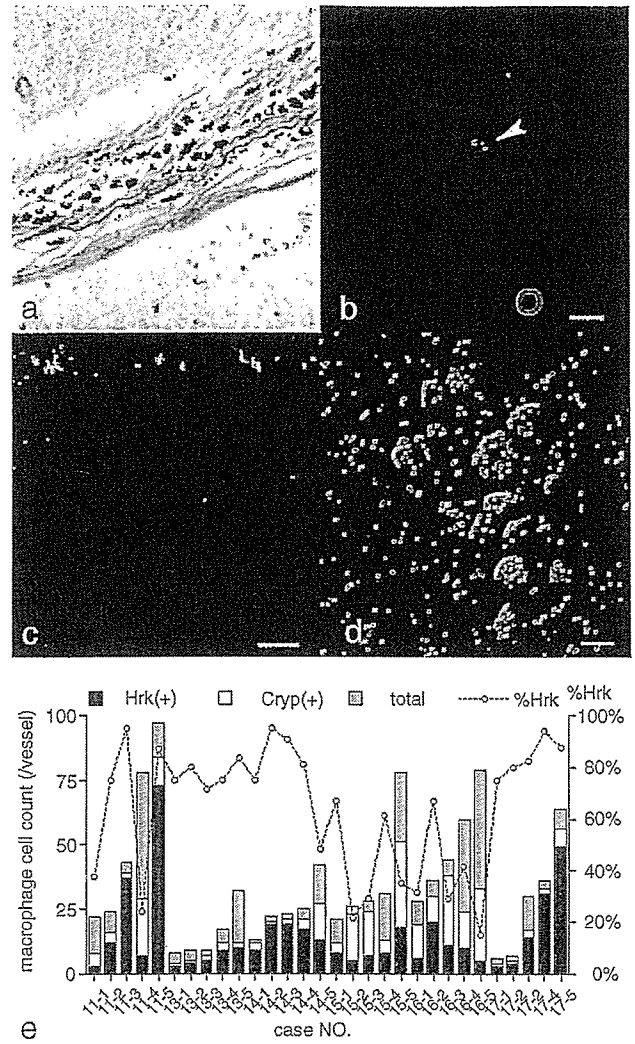

Fig. 2. Immunohistochemistry for infection of the Hrk expressing cells: (a) Grocott staining in the Hrk expressing perivascular cells. Most of the cytoplasmic inclusions were strongly stained with Groco (patient 11: bar = 20 μm); (b) immunoreactivity against *C. neoformans* and HLA-DR (CR3/43). Small circular immunoreactivity against *C. neoformans* (arrow head, Green fluorescence; FITC) was co-localized in the CR3/43 (Red fluorescence; Cy3) expressing macrophages (patient 19: overlay image, bar = 10 μm); (c) The immunoreactivity against Hrk (Green fluorescence; FITC) in a HLA-DR (Red fluorescence: Cy3) presenting cell (patient 12: overlay image, bar = 10 μm) The immunoreacticity agaisnt Hrk was diffusely distributed in the cytoplasm of HLA-DR expressing macrophages (overlay image: bar = 10 μm); (d) Co-localization of HIV p24 and Hrk in the perivascular cells. Small granular immunoreactivity against HIV p24 (red fluorescence; Cy3) was scattered in the Hrk positive (green fluorescence; FITC) perivascular cells (patient 9: overlay image: bar = 20 μm); and (e) the quantitative analysis of Hrk positive cells around cerebral vessels. (A total of six patients were subjected to the analysis. Five vessels were randomly picked up from a patient. Patient's ID numbers (11, 12, 13, 14, 16, 17) and vessel specimen numbers (1–5) are indicated). There is no apparent relationship between the numbers of total infiltrated macrophages (total; blue column) and the number of Hrk(+) macrophages (red column), even in the same patient. The proportion of Hrk expressing cells (Hrk(+)) in *C. neoformans*-positive cells (%Hrk = Hrk(+)/Cryp(+)) is variable in each vessels or patients.

Our results demonstrate that a pro-apoptotic BH3 protein, Hrk, is upregulated in the HIV-1 infected macrophages in HIVE.