難となるなどの運動機能障害が悪化したため再入院となった．再入院時，動作緩慢，失調性歩行で両側バビンスキー徴候陽性，尿失禁をみとめ，HDS-R 19点であった．血液検査はCD4 171/μl，HIVウイルス量は感度以下であった．髄液検査および頭部MRIでは日和見感染症を示唆する所見はみとめず，経過からHIV脳症を当初より合併していたと考えHAARTを継続した．深夜に家族と偽って知人を病室に招き入れるなど病棟のルールを守れずに強制退院となった．その後は精神科外来にて抗躁薬と抗精神病薬を投与し，徐々に落ち着きを取りもどしたが，HDS-Rは20点前後で推移している．

症例4　28歳男性

職業は代用教員．18歳時にバーキットリンパ腫に対し自己末梢血幹細胞移植を受け治癒している．2005年5月ころからものわすれを自覚した．同年6月，バーキットリンパ腫の経過観察のため施行した血液検査で汎血球減少を指摘され，前医に入院しHIV抗体を測定したところ，陽性と判明した．同年7月，職場で倒れているのを発見され救急車にて当院を受診した．体温37.7℃，朦朧状態で物品呼称および理解は比較的保たれているが復唱はできず，上肢の観念運動失行，右同名半盲，右注視麻痺，構音障害，右不全片麻痺，バビンスキー徴候右陽性をみとめた．入院時頭部MRIではT2強調画像にて左右対称性びまん性の白質病変をみとめた(Fig. 2)．入院3日目より右片麻痺，失語は急速に改善し，入院1週間後の診察では失見当識をみとめるが失語や麻痺は消失していた．動作緩慢であり，50音表の書き取りに105秒かかった．MMSEは22点，立方体は模写できなかった．WAIS-Rは言語性IQ 84，動作性IQ 79，全体IQ 80と低下しており，空間能力低下，短期記憶障害などの認知機能障害をみとめた．SPECTでは両側前頭葉の血流低下に加え，左頭頂葉付近の血流増加をみとめ，脳波では左前頭部に棘波をみとめた．運動機能障害と認知機能障害をみとめ，頭部MRIでも白質病変をみとめることから，亜急性にHIV脳症を生じており，今回の入院契機であった一過性の大脳左半球症状はてんかん様発作であった可能性が考えられた．その後外来にてHAARTを施行した．発症より約6カ月後，見当識は良好だが時に単語がすっと出てこないことがある．運動障害はなく，HDS-Rは27点，MMSEは25点，50音表の書き取りは35秒で可能だがラ行が抜けていた．立方体模写は可能となった．HAART前後での頭部MRIを比較すると，わずかに病変は縮小しており，画像検査上もHIV脳症の改善をみとめた．転職し社会復帰を果たしている．

症例5　63歳男性

職業は会社員．2005年8月に微熱と全身倦怠感，体重減少を自覚した．10月初旬より上記に加えて湿性咳嗽，見当識障害，夜間せん妄が出現した．10月中旬に体重減少と呼吸苦の精査にて前医入院し，胸部CTにて間質性肺炎，胃内視鏡下生検にてサイトメガロウイルス胃炎と判明し，HIV抗体陽性であったため当院に転院した．転院時，呼吸不全をみとめ，神経学的診察では，軽度意識障害（Japan Coma Scale-2），自発性低下，動作緩慢をみとめ，指鼻試験および膝踵試験は拙劣で，起居動作や歩行時にはふらつきがあり，つぎ足歩行はできなかった．立方体模写は不可能で，1から26の数唱に26秒，同じく書き取りに36秒かかった．50音表の書き取りは途中で止まってしまい遂行できなかった．全身状態が安定した後に施行したHDS-Rは12点，WAIS-Rは言語性IQ 62，動作性IQ 52，全体IQ 55と著明な低下をみとめた．頭部MRIにて，橋と両側大脳前頭葉白質から脳梁にT2強調画像でびまん性の高信号域をみとめた．当院転院後，ST合剤およびステロイドによるカリニ肺炎の治療を施行し呼吸状態は改善したが，神経学的には変化がなかった．11月よりHAARTを施行したところ直後に一過性の譫妄をきたしたが，開始1週間後より病室で小説を読み，徐々に他の症状も改善していった．HAART開始1カ月後の診察では意識清明，歩行は自立しているがつぎ足歩行は不可能であった．HDS-R 29点，MMSE 30点と改善をみとめた．50音表書き取りは「な」行でとまってしまった．2カ月後に自宅退院され，現在も外来にてHAARTを施行している．

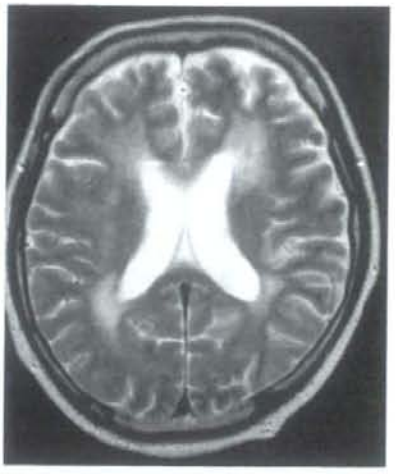

Fig. 2　Brain MRI (T2 weighted image) of case 4
It revealed that the diffuse high intensity area in cerebral white matter spared the subcortex.

考　察

HIV感染症は近年，HAARTをはじめとする治療法の進歩によって当初恐れられていた日和見感染症や悪性腫瘍は減少傾向を辿っており[2,3]，中枢神経合併症も同様の傾向を呈している[4-7]．しかし，HIV脳症はHAARTによっても発症頻度が減少しないとされ[6,8]，その理由としてはHAARTによってHIV脳症をふくめたAIDS症例全体の生命予後が改善することが指摘されている[9]．

今回のHIV脳症5例について，診断時のCD4とHIVウイルス量をTable 2にまとめた．いずれもCD4は200/μl以下

で平均22.4/μlときわめて低値であり，諸外国でのHAART導入以前と同様の傾向を示している[8]．他の日和見感染症を合併しており，高度の免疫不全状態であったと思われた．

次に，HIV脳症診断時の髄液検査所見については，Table 3に示すように症例5を除いていずれも細胞数は正常（症例5についても1週間後の再検査時には正常），蛋白は正常から微増であった．測定しえた症例では，髄液中β-2ミクログロブリンはいずれも2μg/mlを超えていた．髄液中のHIVウイルス量はばらつきが多いものの症例1，3，4では血液中のウイルス量と比較しても高値であった．髄液中の糖は全症例とも低値を示した．HIV脳症において髄液中の細胞数増多や蛋白の上昇がときにみとめられることは知られているが，髄液中の糖についてはあまり検討がなされておらず，Naviaらが HIV脳症41症例中1例のみ糖が低値であったと報告している[1]．われわれが経験した5症例において，頭蓋内の細菌感染症は髄液培養検査が陰性であったことや経過から否定的であり，髄液中の糖が低値であった理由は不明であった．

HIV脳症の症状は運動，認知，行動の3つに大別される[1]．今回の5症例において，運動障害と認知障害は程度の差異はあるものの全症例にみとめられたが，行動異常の有無については症例差がいちじるしかった．運動機能障害については動作緩慢は全症例とも改善をみとめた．失調は完全消失にはいたらないものの改善傾向であり，結果として関節炎による関節拘縮をきたした症例1以外を除いては日常生活動作が自立となっており，運動機能予後は良好と思われた．

認知機能について，経過中に適宜施行したHDS-RもしくはMMSEの結果からはいずれの症例も追跡しえた範囲では改善傾向にあり，症例1は31カ月を経過した時点でもなお改善傾向にあるが，依然障害は残存している．

認知障害とならんで，行動障害は服薬アドヒアランスを大きく低下させ療養を困難とする要因となった．症例1，2，4では経過中に顕著な行動異常が出現し，今回の症例で唯一症例4のみが就労を果たした．他の症例と比較すると認知機能障害の残存はみとめていたものの，診断当初より無気力をはじめとする行動障害をともなっていなかったことがその要因と思われた．このことからは，HIV脳症に特徴とされる運動機能障害，認知機能障害，行動障害のうち，行動障害が強いばあいには就労は困難となりうることが示唆された．

HIV脳症の治療として，全症例ともできるだけ早期にHAARTを導入した[10][11]．今回追跡しえた期間内は死亡をみとめず，他の中枢神経疾患が多くのばあいに致死性である[7][12]ことを考えると，HIV脳症の短期間の生命予後は良好であると思われた．その一方で機能予後は不良と考えられ，HAARTのみでは治療効果は不十分であると思われた．今後，HAARTに加えてあらたな治療の確立が望まれる[13][14][15]．

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Abstract

Clinical features and courses of 5 cases with HIV encephalopathy

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Clinical features and courses of 5 cases with HIV encephalopathy were reported. The subjects were among the patients registered as HIV patients at the Nagoya Medical Center, between 1996 and 2005. There were 458 patients with HIV infection including 127 cases of AIDS. All patients suffered from severe immunological deficiency when HIV encephalopathy developed. Other opportunistic infections had also occurred in three patients. HIV encephalopathy was one of the presenting manifestations of HIV infection in four patients, and no patients had received antiretroviral therapy. HAART improved motor disturbance and their ADL became independent except for one case. Improvements in neuropsychological examination scores were noted in all cases. Recovery from psychiatric symptoms, however, was incomplete. Four patients could not work, and 3 needed psychological treatment due to behavioral abnormalities. HIV encephalopathy is not a lethal disease but the functional prognosis was very poor. New therapy is needed for HIV encephalopathy.

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Key words: HIV, AIDS, cognitive impairment, behavioral change, prognosis

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Different Abilities of Escape Mutant-Specific Cytotoxic T Cells To Suppress Replication of Escape Mutant and Wild-Type Human Immunodeficiency Virus Type 1 in New Hosts▽

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There is much evidence that in human immunodeficiency virus type 1 (HIV-1)-infected individuals, strong cytotoxic T lymphocyte (CTL)-mediated immune pressure results in the selection of HIV-1 mutants that have escaped from wild-type-specific CTLs. If escape mutant-specific CTLs are not elicited in new hosts sharing donor HLA molecules, the transmission of these mutants results in the accumulation of escape mutants in the population. However, whether escape mutant-specific CTLs are definitively not elicited in new hosts sharing donor HLA molecules still remains unclear. A previous study showed that a Y-to-F substitution at the second position (2F) of the Nef138-10 epitope is significantly detected in HLA-A*2402$^+$ hemophilic donors. Presently, we confirmed that this 2F mutant was an escape mutant by demonstrating strong and weak abilities of Nef138-10-specific CTL clones to suppress replication of the wild-type and 2F mutant viruses, respectively. We demonstrated the existence of the 2F-specific CTLs in three new hosts who had been primarily infected with the 2F mutant. The 2F-specific CTL clones suppressed the replication of both wild-type and mutant viruses. However, the abilities of these clones to suppress replication of the 2F virus were much weaker than those of wild-type-specific and the 2F-specific ones to suppress replication of the wild-type virus. These findings indicate that the 2F mutant is conserved in HIV-1-infected donors having HLA-A*2402, because the 2F-specific CTLs failed to completely suppress the 2F mutant replication and effectively prevented viral reversion in new hosts carrying HLA-A*2402.

Cytotoxic T lymphocytes (CTLs) play an important role in the control of human immunodeficiency virus type 1 (HIV-1) replication during acute and chronic phases of HIV-1 infections (9, 28, 34). However, CTLs cannot completely eradicate HIV-1 because HIV-1 escapes from the host immune system by various mechanisms, including mutations of immunodominant CTL epitopes (10–12, 40). A substitution of one amino acid within CTL epitopes is crucial for binding to HLA class I molecules or for the interaction between the T-cell receptors (TCRs) of specific CTLs and the peptide-HLA class I complex. Both mechanisms result in the loss of CTL activities against target cells infected with HIV-1 and contribute to the selection of a virus able to escape from CTLs (10, 13, 23, 26, 35). There are many studies demonstrating that CTL-mediated immune pressure selects CTL escape variants during both acute and chronic HIV-1 and simian immunodeficiency virus (SIV) infections (2, 15, 31) and that selection of the escape mutants could result in the loss of immune control and disease progression (6, 16, 23). The escape of HIV-1 from CTL responses has been proposed to be an important obstacle for HIV-1 vaccine development (7, 16, 39).

HIV-1 mutations that allow escape from HIV-1-specific CTLs are HLA dependent because HIV-1-specific T-cell responses are restricted by HLA alleles. This means that an HIV-1 escape mutant can adapt in populations sharing some dominant HLA alleles (33). An escape mutant can be transmitted vertically from mother to child (21, 22) and horizontally between individuals through unprotected sexual intercourse (USI) (3, 20, 21, 29). A study on HIV-1 evolution has provided direct evidence that an escape mutation of an HLA-B57/5801-restricted CTL epitope is stable after transmission to individuals who did not share HLA-B57/5801 and suggested the accumulation of the escape mutation in the population (29). On the other hand, a recent study demonstrated that an escape mutant selected by the CTLs specific for the wild-type (WT) virus can elicit the escape mutant-specific CTLs in the same donors (4), suggesting the possibility that these escape mutant-specific CTLs are elicited in new donors carrying the same restriction allele. If these escape mutant-specific CTLs are elicited in the donors, it is likely that the escape mutant cannot adapt in them. However, it is well known that in both HIV-1 and SIV infections, common escape mutations are poorly recognized in new hosts who share the same HLA alleles with a donor (17, 32).

In a Japanese population infected with HIV-1 through USI, mutant viruses with Y-to-F substitutions at the second position (2F) in the HLA-A*2402-restricted, Nef138-10 WT CTL epitope (RYPLTFGWCF) were shown to accumulate in HLA-A*2402-positive and even HLA-A*2402-negative patients (20). Nef138-10-specific CTLs are frequently detected in

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chronically HIV-1-infected, HLA-A*2402-positive Japanese individuals (25), suggesting that the Nef138-10 CTL epitope is an immunodominant CTL epitope in the population. On the other hand, the 2F mutation of this epitope impaired the cytotoxic activity of the Nef138-10-specific CTLs, suggesting this mutation to be an escape one (20). We found that Nef138-10 WT tetramer$^+$CD8$^+$ T cells frequently exist, even in HLA-A*2402-positive Japanese patients with primary infections (unpublished data). As most of these Japanese patients were infected with the 2F mutant virus, we speculated that 2F-specific CTLs would be elicited in new hosts having HLA-A*2402.

The present study addressed the following three questions. Do Nef138-10-specific CTLs have strong abilities to suppress HIV-1 replication, but fail to suppress replication of the 2F mutant? Can the 2F escape mutant elicit 2F mutant-specific CTLs in a new host? Can the 2F-specific CTLs suppress replication of 2F mutant and WT viruses? The answers to these are expected to clarify the mechanisms of accumulation of escape mutants in the population.

MATERIALS AND METHODS

Patient samples. This study was approved by the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. Patient HLA types were determined by standard sequence-based genotyping. Donors with structured treatment interruption belonged to the clinical trial designed as a prospective study of the AIDS Clinical Center, International Medical Center of Japan.

Patients with early HIV infection, with or without acute retroviral symptoms, were recruited. Early HIV infection was confirmed within 6 months before recruitment by a documented history of seroconversion detected by enzyme-linked immunosorbent assay or by a longitudinal increase in band density on Western blots. Patients with active opportunistic infections or psychological disorders, or those treated with immunomodulatory agents, were excluded.

Sequence of autologous virus. Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (QIAGEN), and cDNA was synthesized from the RNA with SuperScript RNase H reverse transcriptase and random primer (Invitrogen). The Nef region was amplified by nested PCR using *Taq* DNA polymerase (Promega). Proviral DNA was extracted from HIV-1-infected patient PBMCs by using a QIAamp DNA blood mini kit (QIAGEN). We prepared the following *nef*-specific primer sets: 5′-AGCAGCAGATGGGGTGGGAGC-3′ and 5′-AGCATCTGAGGGACGCCACTCCC-3′ for the first PCR primer set 1, 5′-TCGAGACCTGGAAAAACATGGAGC-3′ and 5′-AAAGTCCCCAGCGGAAAGTCCC-3′ for the second PCR primer set 1, 5′-TCGAGACCTGGAAAAACATGGAGC-3′ and 5′-TAACCAGAGAGACCCAGTACAGGC-3′ for the first PCR primer set 2, and 5′-GGCTAGAAGCACAAGAGGAGG-3′ and 5′-AGCATCTGAGGGACGCCACTCCC-3′ for the second PCR primer set 2. The PCR cycling conditions were 94°C for 30 s, followed by 30 cycles of 30 s at 94°C, 40 s at 55 or 60°C, and 1 min at 72°C, with a final extension of 72°C for 7 min or 94°C for 1 min for the first PCR, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C, with a final extension of 72°C for 7 min for the second PCR. The PCR products were then agarose gel purified and sequenced directly or cloned by the use of a TOPO TA cloning kit (Invitrogen). All DNA sequencing was performed by using a BigDye Terminator (version 1.1) cycle sequencing kit (Applied Biosystems) and an ABI PRISM 310 genetic analyzer.

HIV-1-specific CTL clones. For the generation of KI-158-derived and KI-144-derived CTL clones, PBMCs which were obtained at weeks 69 and 64, respectively, after the first visit were used. Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding cells of this population into U-bottom, 96-well microtiter plates together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human interleukin-2, irradiated allogeneic PBMCs from a healthy donor, and irradiated C1R-A*2402 cells prepulsed with the corresponding peptide), as previously described (25). Nef138-10-specific and Nef138-10-2F-specific CTLs were generated by using WT (RYPLTFGWCF) and 2F (RFPLTFGWCF) peptides, respectively. All CTL clones were cultured in RPMI 1640-10% FCS supplemented with 200 U/ml recombinant human interleukin-2 and stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

HIV-1 clones. Infectious proviral clones of HIV-1, pNL-432 and its Nef mutant pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), reported previously, were used (1). For pNL-432-10F, pNL-432-2F10F, and pNL-M20A-10F, mutations were introduced by site-directed mutagenesis based on overlap extension.

Flow cytometric analysis for surface expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells. HLA-A*2402-positive CD4$^+$ T cells infected with HIV-1 clone were stained to assess the expression of HLA class I in HIV-1-infected CD4$^+$ T cells, as previously described (38). Briefly, the cells were stained with anti-A11 and -A24 monoclonal antibody (MAb) A11.1 M, following staining with phycoerythrin (PE)-conjugated anti-mouse immunoglobulin (Ig) (Dako-Cytomation, Glostrup, Denmark) and thereafter were fixed and permeabilized for intracellular HIV-1 p24 staining with fluorescein isothiocyanate (FITC)-conjugated anti-p24 MAb KC-57 (Beckman Coulter, Miami, FL). The expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells was analyzed by using a flow cytometry.

Peptide-binding assay. The binding of peptides to HLA-A*2402 molecules was tested as previously described (25). HLA-A*2402 RMA-S transfectants, which were transfected with HLA-A*2402 and human β_2-microglobulin, lacked functional TAP2. They express a very low level of HLA class I molecules on the cell surfaces when they are cultured at 37°C, while empty HLA class I molecules are stably expressed if they are cultured at 26°C, because empty HLA class I molecules are unstable on cell surfaces at 37°C but stable at 26°C. The stabilization of HLA class I molecules is dependent on peptide binding affinity (24, 30, 36). Briefly, RMA-S-A*2402 cells were cultured at 26°C for 14 to 18 h. The cells were incubated at 26°C for 1 h with WT (RYPLTFGWCF) or 2F (RFPLTFGWCF) peptide at various concentrations and then at 37°C for 3 h. After two washes with phosphate-buffered saline (PBS) supplemented with 20% FCS (PBS-20% FCS), they were incubated for 30 min on ice with an appropriate dilution of MAb TP25.99. After two washes with PBS-20% FCS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated sheep IgG with anti-mouse Ig specificity (Silenus Laboratories, Hawthorn, Australia). Finally, they were washed three times with PBS-20% FCS, after which the fluorescence intensity was measured by using a flow cytometer (Becton Dickinson, Mountain View, CA).

HLA-peptide tetrameric complexes. The tetrameric complexes were synthesized as previously described (5). Briefly, an ectodomain of HLA class I proteins and human β_2 microglobulin, produced in *Escherichia coli* that had been transformed with the relevant expression plasmids, were first solubilized in denaturing buffer containing 8 M urea and refolded in refolding buffer in the presence of a synthesized peptide for 48 h at 4°C. The resultant 45-kDa complex was purified by size exclusion (Superdex G75; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England) and anion exchange chromatographies (MonoQ column; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE-conjugated avidin (ExtrAvidin-PE; Sigma-Aldrich, St. Louis, MO) at a molar ratio of 4:1.

Tetramer analysis. CTL clones were first stained with either PE-conjugated WT or allophycocyanin (APC)-conjugated mutant (2F) tetramer (final concentration, 300 nM) at 37°C for 30 min. For the competitive assay, the clones were stained with WT and 2F tetramer (final concentration, 300 nM for each tetramer) at the same time at 37°C for 30 min. After two washes with RPMI 1640 medium supplemented with 10% FCS (RPMI 1640-10% FCS), the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min. For ex vivo analysis, thawed PBMCs were stained with PE-conjugated WT and APC-conjugated 2F tetramers (final concentration, 300 nM for each tetramer) at 37°C for 30 min. Following two washes with RPMI 1640-10% FCS, the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min and subsequently analyzed by using flow cytometry. The percentage of tetramer-positive cells among CD8-positive cells was then calculated.

Replication suppression assay. The abilities of HIV-1-specific CTLs to suppress HIV-1 replication were examined as previously described (37). Briefly, CD4$^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C. After three washes with RPMI 1640-10% FCS, the cells were cocultured with HIV-1-specific CTL clones. From day 3 to day 9 postinfection, 10 μl of culture supernatant was collected and the concentration of p24 antigen (Ag) was measured by use of an enzyme immunoassay (HIV-1 p24 Ag enzyme-linked immunosorbent assay kit; ZeptoMetrix Corporation, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells

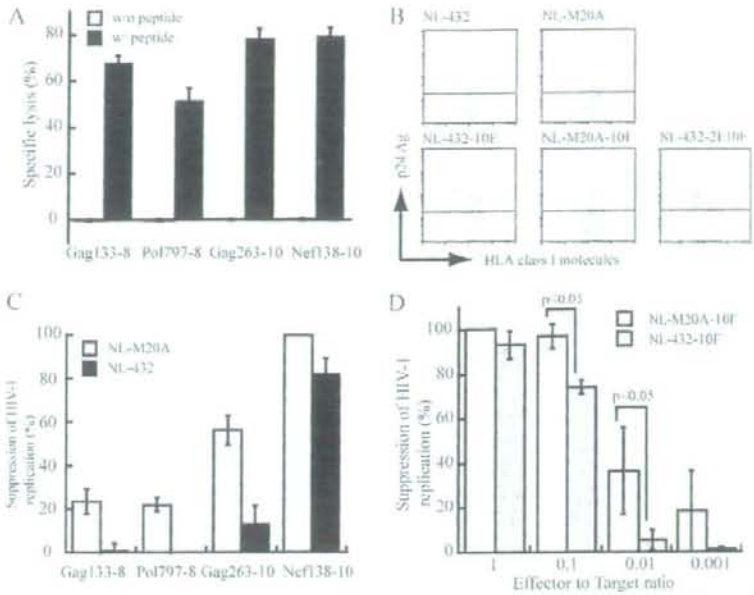


FIG. 1. Suppression of HIV-1 replication in HIV-1-infected CD4$^+$ T cells by HLA-A*2402-restricted HIV-1-specific CTLs. (A) Cytolytic activities of HLA-A*2402-restricted HIV-1-specific CTLs (four Gag133-8-specific, five Pol797-8-specific, four Gag263-10-specific, and four Nef138-10-specific CTL clones) toward HLA-A*2402$^+$ cells pulsed with (w) the indicated peptides (1,000 nM). w/o, without. The cells were tested at an E-to-T ratio of 2:1. Values represent averages ± standard deviations (SD) (error bars) of results from triplicate assays. (B) CD4$^+$ T cells derived from healthy donors were infected with NL-432, NL-M20A, NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cultured for 3 to 5 days. The cultured CD4$^+$ T cells were stained with anti-p24 and A11.1 M anti-A11 and anti-A24 MAbs. (C) Suppression of HIV-1 replication by HLA-A*2402-restricted CTLs. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432 or NL-M20A and then cocultured with HIV-1-specific CTLs at an E-to-T ratio of 1:1. HIV-1 p24 Ags in the supernatant were measured on day 6 postinfection by conducting an enzyme immunoassay. The percentage of suppression of HIV-1 replication was calculated. Values represent averages ± SD (error bars) for four Gag133-8-specific, three Pol797-8-specific, four Gag263-10-specific, and three Nef138-10-specific CTL clones. (D) The ability of Nef138-10-specific CTL clone 189 to suppress NL-432-10F and NL-M20A-10F replication in CD4$^+$ T cells. HIV-1-infected HLA-A*2402$^+$CD4$^+$ T cells were cocultured with Nef138-10-specific CTLs at various E-to-T ratios. Values represent averages ± SD (error bars) of results from two independent experiments. The *P* values were determined by nonparametric Mann-Whitney test.

cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells cultured without the CTLs) × 100.

^{51}Cr release assay. The cytotoxicity of CTL clones against C1R-A*2402 prepulsed with appropriate peptide at various concentrations or HIV-1-infected 221-CD4$^+$-A*2402 cells was determined as previously described (37, 38). Briefly, target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline. Effector cells were cocultured with target cells (2×10^3/well) for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cmp max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100. Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the cpm in the supernatant in the wells containing both the target and effector cells at an effector-to-target ratio of 2:1. The peptide concentration providing half of the maximum percentage of specific lysis (LL_{50}) was calculated by using KaleidaGraph (Hulinks, Inc., Tokyo, Japan).

RESULTS

Complete suppression of HIV-1 replication by HLA-A*2402-restricted Nef138-10-specific CTLs. Since Nef138-10-specific CTLs are frequently detected in HLA-A*2402-positive HIV-1-infected individuals, it has been speculated that Nef138-10 (RYPLTFGWCY) is an immunodominant epitope and that these specific CTLs can strongly suppress HIV-1 replication. To clarify the abilities of Nef138-10-specific CTLs to suppress HIV-1 replication, we compared the abilities of four epitope-specific, HLA-A*2402-restricted CTL clones to suppress HIV-1 replication by assaying for the suppression of HIV-1 replication. All CTL clones specific for the Gag133-8, Pol797-8, Gag263-10, or Nef138-10 epitope showed similar cytotoxic activities against target cells prepulsed with the corresponding peptide (Fig. 1A). Gag133-8, Pol797-8, and Gag263-10 epitopes are conserved in both NL-432 and NL-M20A strains, the latter of which has the ability to down-regulate the expression of CD4 molecules, but not the expression of HLA class I molecules, on the cell surface, whereas the Nef138-10 epitope is not conserved in these strains. The Nef138-10 epitope has a tyrosine instead of phenylalanine at position 10, but Nef138-10 epitope-specific CTLs effectively recognized both peptides, RYPLTFGWCF and RYPLTFGWCY. We investigated HLA-A*2402 down-regulation on CD4$^+$ cells infected with NL-432 or NL-M20A. The down-regulation of HLA-A*2402 was found on CD4$^+$ cells infected with NL-432, but not on those infected with NL-M20A (Fig. 1B). We measured the abilities of these CTL clones to suppress HIV-1 replication in primary CD4$^+$ T cells infected with either HIV-1 clone NL-432 or HIV-1 clone NL-M20A. Nef138-10-specific CTL clones com-

pletely suppressed both NL-432 and NL-M20A replication at an effector-to-target (E-to-T) ratio of 1:1, whereas CTL clones specific for the Gag133-8, Pol797-8, or Gag263-10 epitope partially suppressed NL-M20A replication but failed to suppress NL-432 replication (Fig. 1C), indicating that Nef138-10-specific CTLs have strong abilities to suppress NL-432 replication. We generated mutant viruses, NL-432-10F and NL-M20A-10F, carrying the SF2 strain-derived Nef138-10 epitope because the CTL clones specific for the SF2 strain-derived Nef138-10 epitope (RYPLTF GWCF) had been established. Down-regulation of HLA-A*2402 was found on $CD4^+$ cells infected with NL-432-10F, whereas it did not occur on those infected with NL-M20A-10F (Fig. 1B). The strong ability of Nef138-10-specific CTL clone 189 to suppress the replication of both viruses was also found at an E-to-T ratio of 0.1:1 (Fig. 1D). These results indicate that Nef138-10-specific CTLs had strong abilities to suppress HIV-1 replication, regardless of Nef-mediated down-regulation of HLA class I molecules. A significant difference between the abilities of the Nef138-10-specific CTL clone to suppress NL-432-10F and NL-M20A-10F replication was found at E-to-T ratios of 0.1:1 and 0.01:1, but not at an E-to-T ratio of 1:1 (Fig. 1D), suggesting that the suppressive ability of this CTL clone was minimally affected by Nef-mediated HLA class I molecules.

A previous study demonstrated that the 2F substitution of this epitope is associated with HLA-A*2402$^+$ Japanese hemophiliacs (20). We therefore investigated HLA-A*2402-associated mutations of the Nef138-10 epitope in chronically HIV-1-infected Japanese hemophiliacs and nonhemophiliacs. We sequenced the Nef138-10 epitope and its flanking region from HIV-1 RNA from plasma samples from 41 HLA-A*2402-positive and 22 HLA-A*2402-negative Japanese patients (Fig. 2A). We found mainly three types of mutation at the epitope region: a Y-to-F substitution at the second position (2F), a T-to-C substitution at the fifth position, and an F-to-Y substitution at the tenth position. Only the 2F substitution was significantly associated with HLA-A*2402 (Fig. 2B). Although we detected an I-to-T or I-to-V substitution at the −1 position in the N-terminal flanking region, we could not find any association with HLA-A*2402 or other HLA alleles (data not shown). We also analyzed the 2F substitution in two groups, hemophiliacs and nonhemophiliacs who had become infected through USI. In both groups, the 2F substitution was significantly associated with HLA-A*2402 (Fig. 2C). A previous study showed that the 2F substitution was significantly found in the HLA-A*2402-positive Japanese hemophiliacs but not in HLA-A*2402-positive patients infected through USI (20). Our results confirmed the association of the 2F substitution with HLA-A*2402 in Japanese hemophiliacs but suggested a different conclusion for the Japanese patients infected through USI. The frequency of 2F substitutions was much higher in HLA-A*2402$^-$ patients infected through USI than that in HLA-A*2402$^-$ hemophiliacs (31% versus 0%), suggesting that this mutation had accumulated in the HLA-A*2402$^-$ population.

Antiviral activities of Nef138-10-specific CTLs toward NL-432 and Nef138-10-2F mutant virus. To investigate the effect of the 2F substitution in the Nef138-10 epitope on the antiviral activities of Nef138-10-specific CTLs, we first measured the cytotoxic activity of these cells against WT or mutant epitope peptide-prepulsed cells (Fig. 3A). Four CTL clones derived from an HIV-1-infected Japanese patient (KI-158) showed

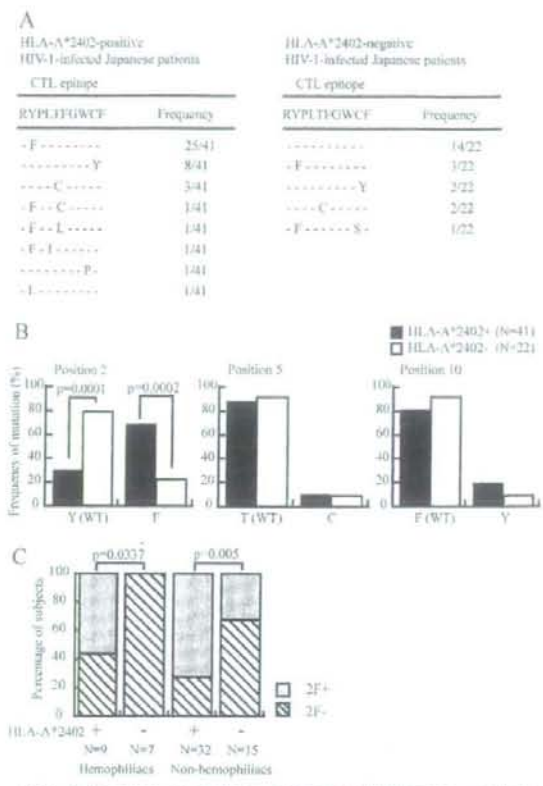


FIG. 2. Frequency of mutation in the Nef138-10 epitope and flanking region. (A) Alignment of the amino acid sequences from 41 HLA-A*2402$^+$ and 22 HLA-A*2402$^-$ patients. (B) Frequency of the substitutions in the Nef138-10 epitope. The Y-to-F substitution at position 2 was more frequently found in the HIV-1-infected Japanese population expressing HLA-A*2402 ($P = 0.0002$). The vertical axis shows the frequency (%) of mutation among 41 HLA-A*2402$^+$ and 22 HLA-A*2402$^-$ patients. The horizontal axis shows the amino acid at each position. (C) Y-to-F substitutions between hemophiliacs and nonhemophiliacs (more than 2 years after infection). Bars indicate the percentages of individuals whose viral sequences are 2F (shaded part of bar) or not (striped part of bar) in subjects either expressing HLA-A*2402 (2F+) or not expressing it (2F−). The P values were determined by Fisher's exact test.

higher cytotoxic activities toward HLA-A*2402$^+$ target cells prepulsed with WT peptide (LL_{50}, 0.16 ± 0.07 nM) than toward those prepulsed with the mutant epitope peptide (LL_{50}, 5.03 ± 6.23 nM). The difference in CTL activity between the two targets varied among the four clones. The binding affinity of the WT peptide for HLA-A*2402 molecules was higher than that of the mutant peptide, but the difference between these peptides was minimal (Fig. 3B). These results indicate that TCRs of Nef138-10-specific CTLs could effectively recognize the 2F mutant epitope. To investigate the effects of the 2F substitution on the abilities of Nef138-10-specific CTLs to recognize target cells infected with HIV-1, we measured the activities of Nef138-10-specific CTL clones to kill HIV-1-infected cells and to suppress HIV-1 replication in HIV-1-infected

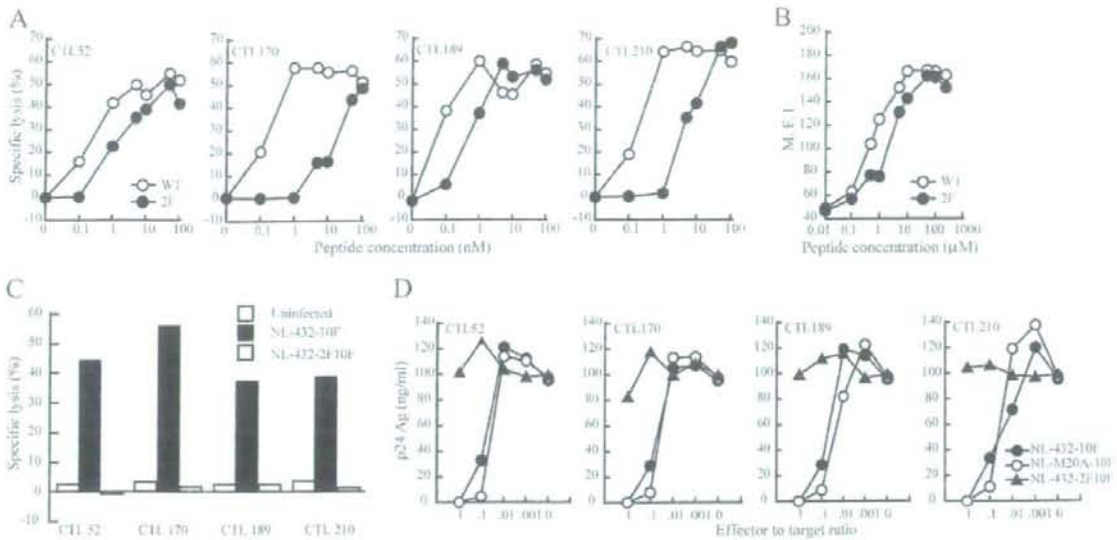


FIG. 3. Abilities of Nef138-10-specific CTLs to suppress replication of NL-432-2F10F. (A) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402$^+$ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). They were tested at an E-to-T ratio of 2:1. (B) Binding of WT and 2F peptides to HLA-A*2402 molecules was quantified by the HLA-A*2402 stabilization assay. (C) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTLs toward NL-432-10F-infected or NL-432-2F10F-infected HLA-A*2402$^+$ cells (40% and 50% p24-positive cells, respectively) used as targets. Cytolytic activity of the CTLs was tested at an E-to-T ratio of 2:1. (D) Abilities of HIV-1-specific CTLs to suppress NL-432-2F10F replication in CD4$^+$ T cells. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cocultured with Nef138-10-specific CTLs at various E-to-T ratios. The HIV-1 p24 Ag level in the supernatant was measured on day 6 postinfection by enzyme immunoassay.

CD4$^+$ T cells (Fig. 3C and D). For the assay, NL-432-2F10F, carrying a Y-to-F substitution at the second position, had been established. All four CTL clones strongly lysed NL-432-10F-infected cells but not NL-432-2F10F-infected cells (Fig. 3C). In addition, those CTL clones strongly suppressed the replication of both NL-432-10F and NL-M20A-10F, but not that of NL-432-2F10F (Fig. 3D). Taken together, these results indicate that the 2F substitution is a mutation permitting escape from Nef138-10-specific CTLs.

Antiviral response to the 2F mutant of CTLs having high-affinity TCRs for the 2F mutant epitope. Among the established Nef138-10-specific CTL clones, we found three CTL clones (from HIV-1-infected patient KI-144) to have much higher cytotoxic activity toward cells prepulsed with mutant peptide than toward those prepulsed with the WT peptide (Fig. 4A). The cytotoxic activities of these CTL clones toward the former were approximately 100-fold higher than those toward the latter (Fig. 4A), suggesting that these CTL clones carried TCRs that more effectively recognized the mutant epitope than that of the WT. To test this possibility, we generated HLA-A*2402 tetramers with Nef138-10 and its 2F mutant and then tested the abilities of these tetramers to bind to the CTL clones at different concentrations of the tetramers. Clone 82 exhibited stronger affinity for the 2F tetramer than for the WT tetramer, whereas clone 189 showed weaker affinity for the former tetramer than for the latter one (Fig. 4B). These results indicate that clone 82 had a TCR with higher affinity for the 2F mutant than for the WT.

It is speculated that CTLs carrying high-affinity TCRs for the 2F mutant would have the ability to recognize cells infected with the 2F virus. Therefore, we investigated the activities of clones 82, 98, and 108 against HIV-1-infected cells by measuring their cytotoxic activities toward HIV-1-infected cells. These CTL clones killed NL-432-2F10F-infected cells (Fig. 4C), indicating that the Nef138-2F mutant epitope was presented in HIV-1-infected cells. Interestingly, they killed NL-432-10F-infected cells more effectively than they did NL-432-2F10F-infected cells (Fig. 4C). This finding indicates that the presentation of the 2F epitope was much weaker than that of the WT one. The results may not reflect Ag presentation of the 2F mutant epitope in HIV-1-infected CD4$^+$ T cells in vivo, because 221 transfectants highly expressing HLA-A*2402 were used as target cells in this assay. We therefore tested the abilities of these CTL clones to suppress HIV-1 replication. They significantly suppressed the replication of NL-432-2F10F at an E-to-T ratio of 1:1 (Fig. 4D and E) and more effectively suppressed the replication of NL-432-10F and NL-M20A-10F than that of NL-432-2F10F (Fig. 4D), supporting the idea that the 2F mutant epitope was presented more weakly than the WT epitope was. Since KI-144-derived CTL clones had higher-affinity TCRs for the 2F mutant and suppressed the replication of NL-432-2F10F, we assume that the 2F mutant-specific CTLs had been elicited by priming with the mutant epitope in this patient.

Ex vivo analysis of the 2F mutant-specific CTLs in HIV-1-infected individuals who had been infected with the 2F mutant virus. Since the 2F mutant-specific CTL clones were established from patient KI-144, we assumed that the 2F mutant-

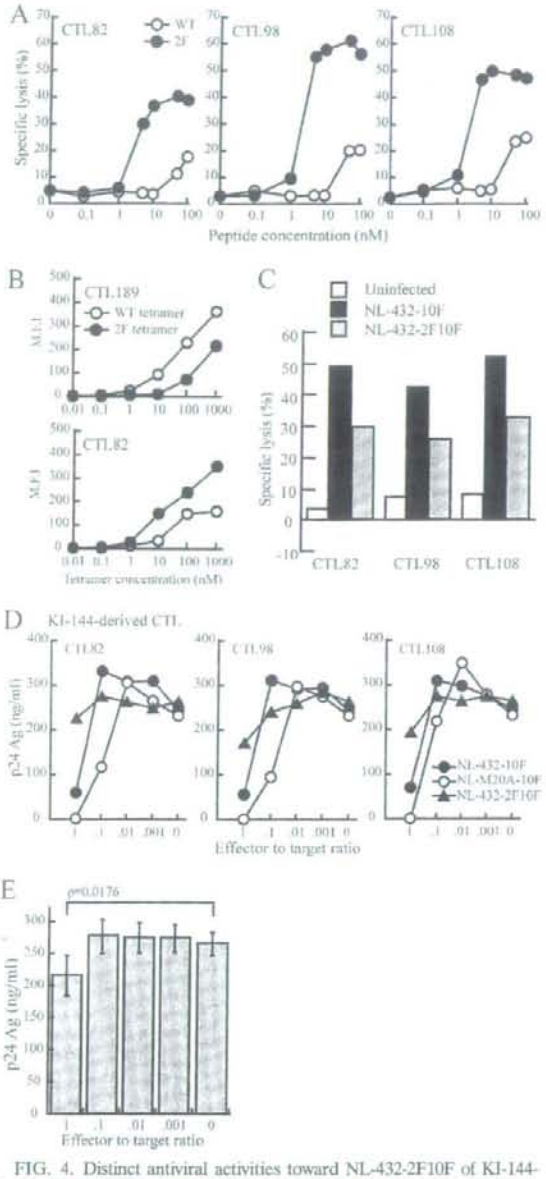
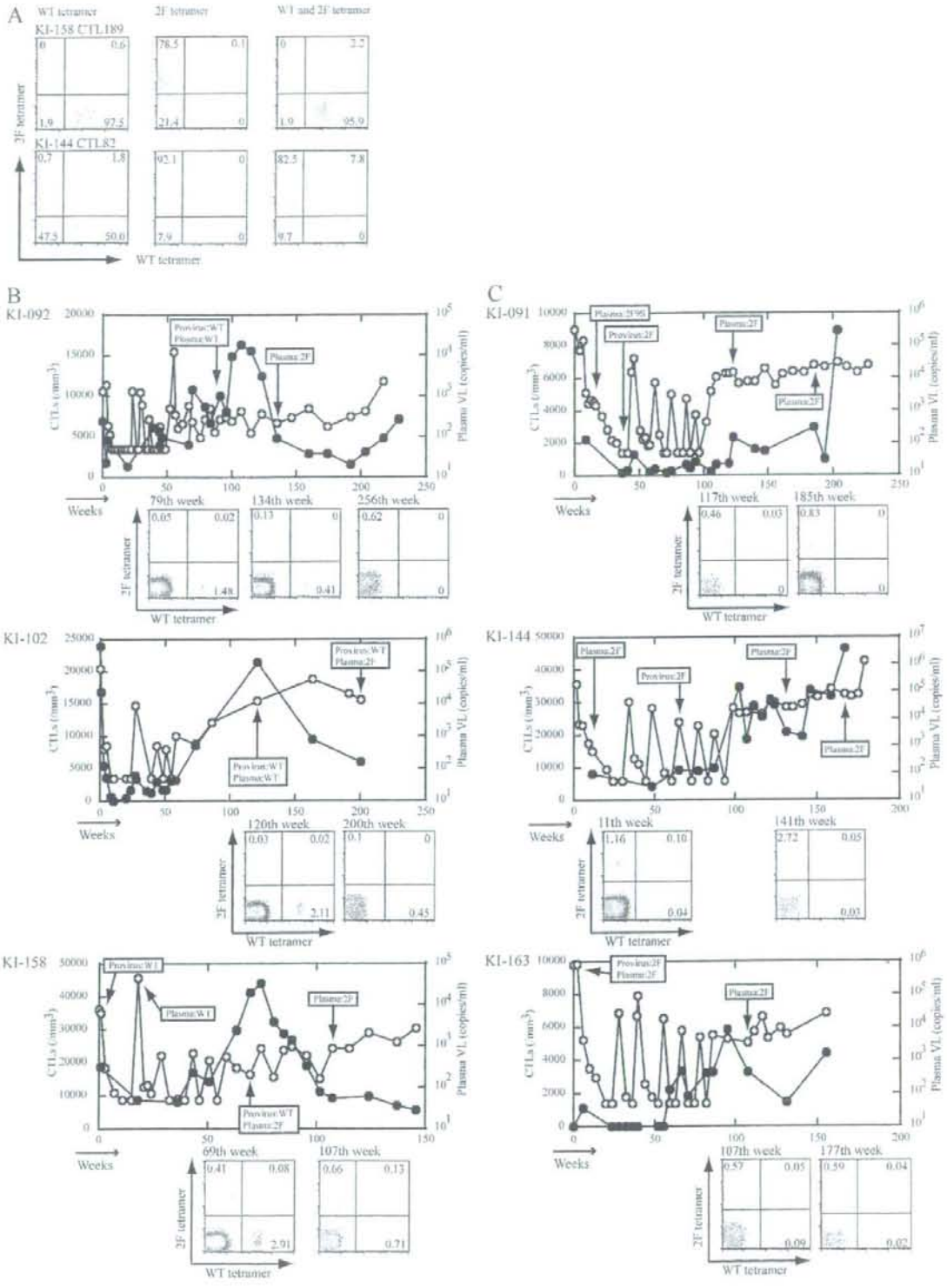


FIG. 4. Distinct antiviral activities toward NL-432-2F10F of KI-144-derived Nef138-10-specific CTL clones. (A) Cytolytic activity of KI-144-derived HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402$^+$ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). The clones were tested at an E-to-T ratio of 2:1. (B) WT and 2F tetramer binding activities of CTL clones 189 (top) and 82 (bottom) clones were determined. (C) Cytolytic activity of clones in panel A toward NL-432-10F-infected or NL-432-2F10F-infected HLA-A*2402$^+$ cells (45% and 55% p24-positive cells, respectively) used as target cells. Cytolytic activity was tested at an E-to-T ratio of 2:1. (D) Abilities of HIV-1-specific CTLs to suppress NL-432-2F10F replication in CD4$^+$ T cells. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cocultured with KI-144-derived CTLs dominantly binding 2F tetramers. The various E-to-T ratios are indicated. The HIV-1 p24 Ag level in the supernatant was measured on day 6 postinfection by using the enzyme immunoassay. (E) Replication of NL-432-2F10F at each E-to-T ratio. Values represent the averages for the three CTL clones ± standard deviations (error bars). Significant differences between cultures with and without CTLs are shown (nonparametric Mann-Whitney test).

specific CTLs were effectively elicited in this patient. To clarify this point, we investigated when and how many 2F-specific CTLs were elicited in this patient. First we established a competitive tetramer binding assay using two HLA-A*2402 tetramers carrying the 2F and the WT peptides to detect the respective 2F-specific and the WT-specific CD8$^+$ T cells. When the CTL clones were stained with either tetramer at different concentrations, clone 82 from patient KI-144 exhibited stronger affinity for the 2F tetramer than for the WT one, whereas clone 189 from patient KI-158 showed weaker affinity for the former tetramer than for the latter (Fig. 4B). Therefore, we expected that each CTL clone would bind to only its higher-affinity tetramer when both tetramers were used at the same concentration. Indeed, when clones 189 and 82 were stained using these tetramers, clones 189 and 82 bound to only the WT and the 2F tetramers, respectively (Fig. 5A). By employing this assay, we investigated the appearance of Nef138-10-specific or 2F-specific CTLs in patients with early HIV-1 infection (Fig. 5B and C). Ex vivo analysis of PBMCs from three patients, KI-092, KI-102, and KI-158, who had been infected with the WT virus, showed that they had mainly the WT-specific CTLs when the WT was found, and then 2F-specific CTLs became predominant after the 2F mutant took the place of the WT-specific CTLs (Fig. 5B). WT virus was found in KI-092 and KI-102 for approximately 2 years after infection, indicating that the 2F mutant had been slowly selected by the specific CTLs. On the other hand, ex vivo analysis of PBMCs from patients KI-091, KI-144, and KI-163 revealed that they had the 2F-specific CTLs only (Fig. 5C). In addition, these patients had 2F sequences in both plasma RNA and proviral DNA throughout their clinical course (Fig. 5C). These findings strongly suggest that these patients had been infected with the 2F mutant. Thus, the 2F mutant-specific CTLs were effectively elicited in donors who had been primarily infected with the 2F mutant virus. However, it remains possible that the number of WT-specific CTLs was too low to be detected by the ex vivo tetramer binding assay. To exclude this possibility, we investigated whether the patients had the WT-specific memory CD8$^+$ T cells. We stimulated their PBMCs with either WT or mutant peptide and cultured the PBMCs for 2 weeks. The WT tetramer-binding CD8$^+$ T cells were then counted by using the competitive tetramer binding assay. The WT-specific CD8$^+$ T cells were not detected among the cultured cells (data not shown), indicating that these patients did not have WT-specific CTLs. These results indicate that when the 2F mutant virus infects a new HLA-A*2402$^+$ host, this host recognizes the 2F mutant epitope and elicits the 2F mutant-specific CTLs.

DISCUSSION

A previous study showed that the 2F mutant was frequently detected in chronically HIV-1-infected individuals having


A
WT tetramer
2F tetramer
WT and 2F tetramer
KI-158 CTL189
KI-144 CTL82
2F tetramer
WT tetramer
B
KI-092
Provirus:WT
Plasma:WT
Plasma:2F
CTLs (/mm3)
Plasma VL (copies/ml)
Weeks
79th week
134th week
256th week
KI-102
Provirus:WT
Plasma:2F
Provirus:WT
Plasma:WT
120th week
200th week
KI-158
Provirus:WT
Plasma:WT
Plasma:2F
Provirus:WT
Plasma:2F
69th week
107th week
C
KI-091
Plasma:2F
Provirus:2F
Plasma:2F
Plasma:2F
117th week
185th week
KI-144
Plasma:2F
Provirus:2F
Plasma:2F
Plasma:2F
11th week
141th week
KI-163
Provirus:2F
Plasma:2F
Plasma:2F
107th week
177th week

HLA-A*2402 and that Nef138-10-specific CTLs failed to kill target cells infected with HIV-1 recombinant Sendai virus containing the 2F mutant; data suggested that the 2F is a mutation for escape from the specific CTLs (20). However, the question remained as to whether Nef138-10-specific CTLs can mediate strong immune pressure on HIV-1 replication so that they select the 2F mutant in vivo. In the present study, we clarified this question by investigating the abilities of Nef138-10-specific CTL clones to suppress replication of the WT and the 2F mutant viruses. Each Nef138-10-specific CTL clone exhibited a strong ability to suppress replication of Nef$^+$ HIV-1 at E-to-T ratios of 1:1 and 0.1:1. This ability is much stronger than that of most HIV-1-specific CTLs (19, 37, 38), suggesting that these CTLs can mediate strong immune pressure in vivo. In addition, our study using the HIV-1 2F mutant showed that Nef138-10-specific CTLs failed to kill target cells infected with the 2F mutant and to suppress replication of the mutant, confirming that 2F is an escape mutant.

Escape mutations occur at sites within CTL epitopes, where the substitution of an amino acid abrogates HLA binding, reduces the recognition of the TCR, and/or interferes with efficient Ag processing (14, 41). The 2F mutant peptide bound to HLA-A*2402 molecules with an efficiency similar to that of the WT peptide. Both the WT and the 2F tetramers bound to Nef138-10-specific CTL clones. In addition, Nef138-10-specific CTL clones killed target cells prepulsed with Nef138-10-2F peptide. These findings suggest that the escape mechanism of the 2F mutant involves the disruption of cellular processing of the 2F mutant peptide. However, since Nef138-10-2F-specific CTL clones effectively killed the target cells infected with the 2F mutant virus and suppressed the replication of the mutant virus, the 2F peptide can be naturally processed and presented by HLA-A*2402. The 2F-specific CTL clones could recognize the 2F peptide much more effectively than the WT peptide, whereas the clones showed much stronger abilities to suppress replication of the WT virus than that of the 2F mutant virus. These observations indicate that the change from Tyr to Phe remarkably reduced the presentation of the epitope peptides in Ag processing but that the 2F mutant could still be presented in the cells infected with the mutant.

It is well known that in both HIV-1 and SIV infections, escape mutations are poorly recognized in new hosts who share the same HLA alleles with donors (17, 32). If escape mutant peptides fail to bind to HLA class I restriction molecules or the mutation critically affects the Ag processing, these escape mutants are hardly recognized and fail to elicit the specific CTLs in new hosts sharing the same HLA alleles. On the other hand, if escape mutant peptides can bind to HLA class I restriction molecules and can be processed and presented, it remains possible that the mutant epitope is recognized in new hosts. The 2F mutant peptide effectively bound to HLA-A*2402 (Fig. 3B), suggesting the possibility that the 2F mutant peptide is presented by HLA-A*2402. We therefore selected the three donors who were infected with the 2F mutant virus at an early phase (within 10 weeks before the first visit) and investigated whether the 2F-specific CD8$^+$ T cells were elicited in these donors. It was strongly suggested that these patients had been infected with the 2F virus, since in the donors who had been infected with the WT virus, the 2F mutation was selected approximately 2 years after infection. The 2F-specific CD8$^+$ T cells were elicited in these three donors, although this mutant epitope was very weakly presented by HLA-A*2402. Thus, escape mutant-specific CTLs can be elicited in new hosts even if the mutant epitope peptide is very weakly presented.

The reversion of a CTL escape mutation to the WT occurs when the mutant virus is transmitted to a new host not sharing HLA class I alleles (18, 29) and even to a new host sharing HLA class I alleles with the monkey donors before the specific CTL is elicited (8, 27). Although the reversion of the 2F epitope to the WT one was reported for chronically HIV-1-infected individuals having no HLA-A*2402, the rate of reversion was very low (20), suggesting that the Y-to-F substitution does not inflict a large fitness cost on HIV-1. A previous study showed that the 2F mutant was still detectable in 56% of HLA-A*2402$^-$ Japanese patients infected through USI (20). In contrast, in the present study, it was found in only 31% of the patients. This difference between these two studies may have resulted from the difference in the time when the sequence was analyzed after the infection. Although the frequency of the 2F mutant in HLA-A*2402$^-$ Japanese individuals is different between the two studies, the studies indicate that 2F mutant did accumulate in HLA-A*2402$^-$ Japanese individuals infected through USI. The reversion of this epitope should occur but may be very slow in HLA-A*2402$^-$ donors. It is thought that the reversion does not occur in HLA-A*2402$^+$ individuals, because the 2F-specific CTLs can strongly suppress replication of the WT virus. Indeed, the reversion was not found in three patients who had been primarily infected with the 2F mutant virus and monitored for 2 to 3 years. Thus, the 2F mutant is accumulating in the Japanese population, of which 70% carry HLA-A*2402.

The competitive tetramer binding assay using the two tetramers could distinguish CD8$^+$ T cells carrying high-affinity TCRs for the WT epitope from those carrying high-affinity ones for the 2F epitope. By using this assay, we found that patients who had been infected with the WT virus first produced WT-specific CD8$^+$ T cells and then 2F-specific CD8$^+$ T cells approximately 6 to 12 months after the 2F mutant had become predominant. In those patients, the 2F mutant virus appeared more than 12 months after the WT virus infection. These findings support our contention that 2F is an escape mutant and that the three donors who had 2F sequences in

FIG. 5. Detection of Nef138-10-2F-specific CTLs in HIV-1-infected patients who had been infected with 2F mutant virus. (A) Tetramer binding of Nef138-10-specific CTLs. KI-158-derived CTL clone 189 (top) and KI-144-derived CTL clone 82 (bottom) were stained with either WT (left panels) or 2F (middle panels) tetramers or both (right panels). The percentage of tetramer-positive cells among CD8$^+$ cells was measured. (B and C) Ex vivo analysis of Nef138-10-specific and Nef138-10-2F-specific CTLs. Nef138-10-specific CTLs in PBMCs derived from HLA-A*2402$^+$ HIV-1-infected individuals were measured by using both WT and mutant (2F) tetramers. Symbols: ●, WT tetramer-positive cells; ○, plasma viral loads. The epitope sequences from viral RNA (plasma) or provirus DNA (PBMCs) during the clinical course are shown. The *x* axis represents weekly course from the first visit. The frequency of CD8$^+$ cells positive for each or both tetramers is given in the quadrants below the graphs. VL, viral load.

their plasma RNA and proviral DNA during the early phase were primarily infected with 2F virus.

In the present study, we demonstrated that new hosts could effectively produce the 2F escape mutant-specific CTLs, even though the 2F mutant epitope was very weakly presented by HLA-A*2402 in HIV-1-infected cells. The 2F-specific CTLs could suppress replication of the 2F mutant virus, but this ability was much weaker toward the 2F mutant than toward the WT virus. The reversion from 2F to WT was not found in the three patients who had been infected primarily with the 2F mutant virus and monitored for 2 to 3 years. This lack of reversion is explained by the fact that the 2F-specific CTLs could effectively suppress replication of the WT virus. This mutant accumulated in HLA-A*2402$^-$ USI patients. Since HLA-A*2402 is a common allele found in approximately 70% of the Japanese population, the 2F mutant can accumulate in the Japanese AIDS population.

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Short Communication

Effects of Low HIV Type 1 Load and Antiretroviral Treatment on IgG-Capture BED-Enzyme Immunoassay

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ABSTRACT

The IgG-capture BED-enzyme immunoassay (BED-CEIA) is used widely at present to detect recent HIV-1 seroconversion. However, antibody levels and antibody kinetics are impacted by HIV-1 load and antiretroviral treatment, which may have a significant effect on the assay results. In this study, we analyzed serial samples from 11 patients with recent infection, including four patients treated by structured treatment interruption (STI), and compared the results with those of 10 untreated and 7 treated patients with chronic infection. The BED-CEIA missidentified one long-term nonprogressor hemophiliac with an extremely low HIV-1 load and five patients with chronic infection who received antiretroviral treatment. We also found that the ODn values increased slowly in patients with recent infection and low HIV-1 loads and that the ODn values fluctuated in parallel with HIV-1 load during STI. Our data indicate that the results of BED-CEIA are influenced by HIV-1 load and antiretroviral treatment. Care should be taken when interpreting the results of BED-CEIA, especially in individuals with low HIV-1 loads. Those on antiretroviral treatment should be excluded from BED-CEIA testing to improve the predictive value of detecting recent infections.

According to the Committee on HIV/AIDS Trends (the Ministry of Health, Labor, and Welfare of the Japanese government), the number of newly diagnosed cases of HIV-1 infection in Japan is continuously increasing and the most frequent mode of transmission is homosexual contact among men who have sex with men.[1] Assessing the incidence of recent infection is important to monitor the current HIV-1 epidemic, although the diagnosis of recent infections usually requires longitudinal follow-up. A new immunoglobulin G (IgG)-capture BED-enzyme immunoassay (BED-CEIA) (Calypte Biomedical Corp., Rockville, MD) was developed recently to identify recent HIV-1 infections.[2,3] BED-CEIA measures the proportion of HIV-specific IgG in serum or plasma samples, which increases after seroconversion. In brief, plates coated with goat antihuman IgG are used to capture both HIV-specific and non-HIV-IgG in test samples. The HIV-specific IgG is detected by a branched multisubtype gp41 peptide labeled with biotin. Incubation with streptavidin-peroxidase followed by tetramethylbenzidine (TMB) substrate allows colorimetric detection of HIV-IgG. The optical density (OD) values of test specimens are normalized (ODn) relative to the value of a calibrator (specimen OD/calibrator OD) to minimize interrun variations. According to the instructions provided by the manufacturer, an ODn of 0.8 corresponds to a mean seroconversion duration of 153 days and the samples with an ODn of <0.8 are considered to be from individuals with recent infection.[4] To assess the reliability of BED-CEIA, we used multiple samples from 28 HIV-1 subtype B-infected patients after obtaining written informed consent.[1]

First, we analyzed the samples of 11 patients with recent infection, 10 untreated patients with chronic infection, and 7 treated patients with chronic infection. The diagnosis of recent infection was made based on the increasing bands of Western blotting against HIV-1 antigens and the used samples were taken at the first visit. The BED-CEIA ODn values of all the 11 samples of recent infection were <0.8 (mean 0.118, SD 0.124) and were correctly identified as recent infection (Fig. 1). Blood samples were also taken from 10 antiretroviral treatment-naive patients with chronic infection (more than 2 years after the first visit). The ODn values of nine of these samples were

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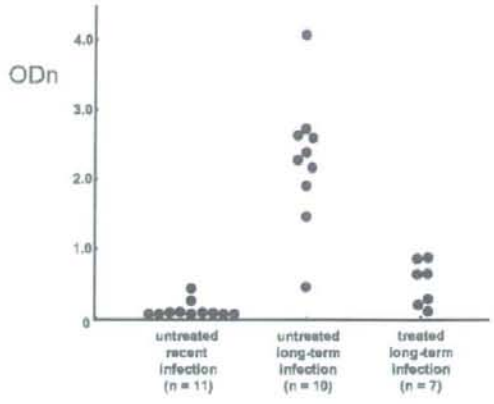

FIG. 1. ODn values of BED-CEIA in three groups of cases. Shown are the ODn values of untreated patients with recent HIV-1 infection and untreated and treated patients with chronic HIV-1 infection.

>1.4 and were correctly identified as long-term infection (mean 2.462, SD 0.718). One sample, however, was missidentified as recently infected (ODn = 0.447), which was taken from a long-term nonprogressor hemophiliac carrying an undetectable HIV-1 load (<50 copies/ml) who had not received antiretroviral treatment and who had acquired his HIV-1 infection before 1985.[5] Blood samples were also taken from seven patients with long-term infection who had received antiretroviral treatment and whose viral load had been persistently suppressed below the detection limit for more than 2 years. Their BED-CEIA ODn values (mean 0.508, SD 0.320) were lower than those of untreated patients with chronic infection ($p = 0.0003$), and five of the seven were incorrectly labeled as recently infected, indicating that antiretroviral treatment negatively alters the reliability of BED-CEIA.

Next, we analyzed serial samples from the same patients to determine the longitudinal changes in ODn. Four of the 11 patients described above with recent infection were subsequently treated with structured treatment interruption (STI), which involves repeated cycles of treatment and interruption intended to evoke a host immune response against HIV-1.[6] We compared the ODn values of these patients with those of the other seven patients who did not receive any treatment to define the natural change after recent infection (Fig. 2). In four patients with logarithmic averages of an HIV-1 load of $\geq 2.0 \times 10^4$ copies/ml, the ODn values gradually increased and all the samples taken more than 153 days after the first visit were correctly identified as long-term infection. However, in the other three patients whose logarithmic averages of HIV-1 load were $\leq 2.8 \times 10^3$ copies/ml (low viral load), the ODn values increased more slowly and many samples were misslabeled as recent infection although they were taken more than 1 year after the first visit. We also analyzed serial samples taken from the treatment-naive long-term nonprogressor hemophiliac described above, and, surprisingly, found a slow increase in the ODn value (Fig. 3). The ODn value of a sample taken in 2005, more than 20 years

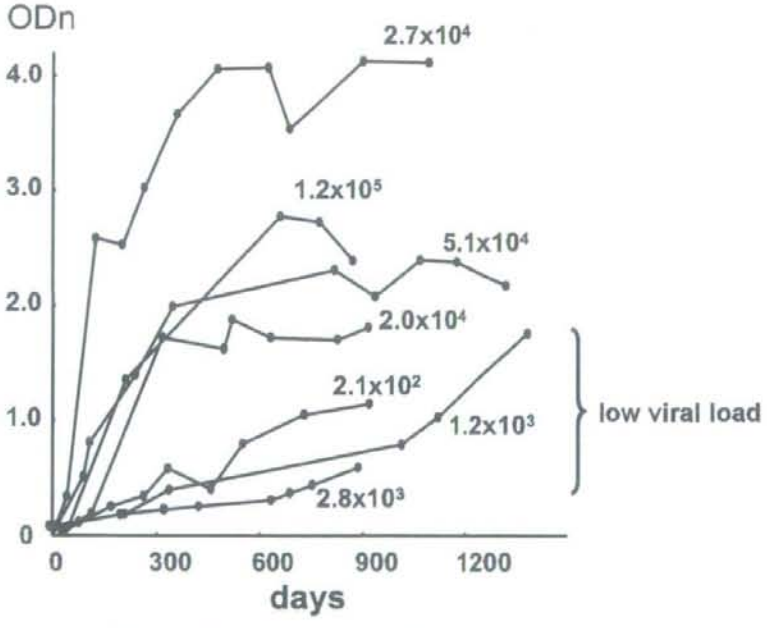

FIG. 2. Serial changes in ODn values of untreated patients with HIV-1 infection. Lines indicate serial changes in ODn values of seven patients with recent infection including three cases with low viral loads.

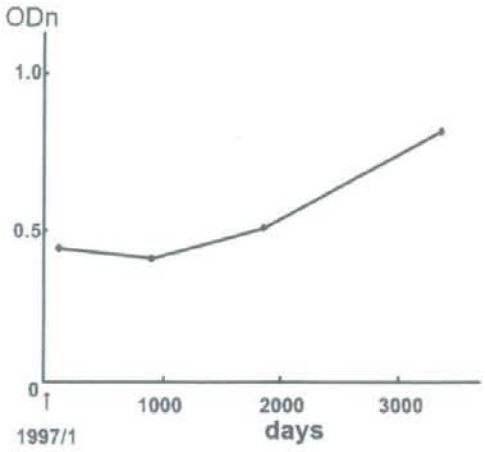

FIG. 3. Serial change in ODn values of one long-term nonprogressor hemophiliac. The line indicates serial changes in ODn values of a long-term nonprogressor with persistently undetectable HIV-1 load who acquired infection before 1985 and had not received antiretroviral treatment. Day 0 is the date of his first visit in January 1997.

after acquiring HIV-1 infection, was 0.865. These data indicate that the ODn values of patients with a low HIV-1 load increase slowly and false-positive recent infection can occur in such cases.

Finally, we analyzed serial samples of four patients with recent infection who received antiretroviral STI therapy to determine the effect of such treatment on ODn values. Figure 4 shows the changes in HIV-1 load and BED-CEIA ODn values of one patient, in whom conventional continuous antiretroviral treatment was administered 462 days after the completion of STI. After the introduction of the first course of antiretroviral treatment, the HIV-1 load sharply decreased and ODn values were persistently low (<0.20). Following a drop in HIV-1 load to below the detection limit (<50 copies/ml), treatment was interrupted for 26 days, during which the HIV-1 load rebounded accompanied by an increase in ODn to 0.760. Then the second course of treatment was introduced, which resulted in a fall of HIV-1 load (400 copies/ml) and ODn value (0.482). The fluctuation in ODn value paralleled the HIV-1 load during STI. Similar data were obtained from the other three cases treated with STI. Considered together, these findings indicate that introduction of antiretroviral treatment resulted in a rapid fall in BED-CEIA ODn values, probably due to antiretroviral treatment-induced suppression of HIV-1 load, suggesting that ODn value are sensitive to changes in HIV-1 load.

Recently, BED-CEIA has been used in a number of cross-sectional populations to estimate incidence and showed excellent results.[3,7] There was plausible agreement between the observed and BED-CEIA-estimated incidence with specimens obtained from a longitudinal cohort study[8] and there was no misclassification of 70 pregnant women with known long-term infection.[3] However, missidentification of long-term infection as recent infection can happen in some cases.[4] Our study indicates that such missidentification is associated with low HIV-1 load and use of antiretroviral treatment, which is consistent with a previous report.[4]

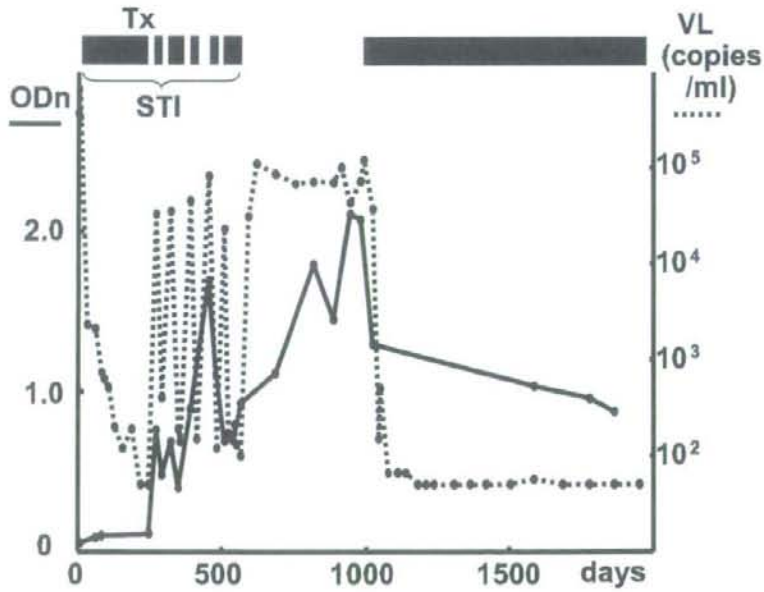

FIG. 4. Effect of antiretroviral treatment on ODn values. Bold and dotted lines show changes in ODn values and HIV-1 load, respectively, in a patient who received STI followed by conventional antiretroviral treatment.

Therefore, whenever possible, those on antiretroviral treatment should be excluded from BED-CEIA testing to improve the predictive value of detecting recent infections.

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CTL-Mediated Selective Pressure Influences Dynamic Evolution and Pathogenic Functions of HIV-1 Nef[1]

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HIV-1 Nef plays multiple roles in modulating immune responses, even though it is a dominant CTL target itself. How Nef accomplishes the balance between such conflicting selective pressures remains elusive. By genetic and functional studies, we found that Arg^{75}Thr and Tyr^{85}Phe mutations, located in a well-conserved proline-rich region in Nef, were differently associated with escape from CTL responses specific for two overlapping HLA-B35-restricted epitopes. CTLs specific for an epitope, that selected Tyr^{85}Phe, were elicited earlier and had more potent functional avidities than did those that selected Arg^{75}Thr. Although the double mutant could escape from both CTLs, the mutations are rarely observed in combination naturally. Introduction of both mutations reduced Nef's HLA class I down-regulation activity and increased the susceptibility of virus-infected cells to recognition by CTLs targeting other epitopes. Moreover, the mutant Nef was impaired in the association with activated cellular kinases and in the enhancement of viral replication. These results highlight CTL immunosurveillance as important modulators of Nef's biological activity in the infected host. *The Journal of Immunology,* **2008, 180: 1107–1116.**

The accessory gene product Nef is a critical determinant for the pathogenesis of the primate lentiviruses, HIV-1, HIV-2, and SIV. The importance of Nef in viral pathogenesis was first shown in rhesus macaques, where a large deletion of the *nef* gene severely reduced SIV pathogenicity (1). This finding was supported by the fact that a cohort consisting of one blood donor and eight transfusion recipients infected with Nef-defective HIV-1 demonstrated dramatically decreased rates of disease progression (2, 3). The impact on the outcome of HIV/SIV infection likely results from the synergy of multiple functions exerted by Nef that may be differentially regulated over time (4). Nef enhances viral replication and virion infectivity (5–7) and affects cells in many ways, including altering T cell activation and maturation (6, 8–11), subverting the apoptotic machinery, and down-regulating a number of cell surface receptors including CD4 and HLA class I (7, 12, 13). The down-regulation of MHC class I (MHC-I)[3] by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo, highlighting the importance of Nef-mediated immunoevasion to facilitate disease progression (14).

The initial peak of viral replication after primary HIV infection begins to decline simultaneously with the appearance of HIV-specific CD8 T lymphocytes (15, 16) that can eliminate HIV-infected cells directly by MHC-I-restricted cytolysis or indirectly through the production of soluble factors such as cytokines and chemokines (17, 18). The biological relevance of HIV-specific CTLs in HIV infection is also supported by the results of in vivo studies demonstrating a dramatic rise of viremia and an accelerated clinical disease progression in SIV-infected macaques after the artificial depletion of $CD8^+$ cells (19, 20). Among HIV proteins targeted by HIV-specific CTLs, HIV Nef protein is expressed at high levels early in an HIV infection (21) and elicits a strong CTL response in a number of subjects (22, 23). Most antigenic determinants are located within a multirestricted, immunodominant central region spanning residues 73–94 and 113–147 (22, 24), including a highly conserved proline-rich region containing an Src homology 3 (SH3)-binding motif, PxxP (Nef_{73-82}: PVRPQVPLRP) critical for several but not all Nef functions (6, 7, 25–27). In particular, HIV-infected subjects expressing the HLA-B*3501 molecule, which prefers a proline residue on the second position of its antigenic peptides, show vigorous HLA-B35-restricted CTL responses toward the proline-rich region of Nef (22, 28, 29).

In the present study, we focused on HLA-B35-restricted CD8 T cell responses toward the functionally important PxxP region of HIV-1 Nef to ask whether CTL responses can impose constraints on Nef activity. Remarkably, sequence analysis of autologous viruses revealed the association of two different mutations with patients carrying HLA-B*35, one of which was earlier shown to be a naturally occurring variation that can modulate Nef functions (25). Further detailed analyses of CTL responses and Nef functions demonstrated that Nef balances between the conflicting selective pressures during the course of an HIV-1 infection. These findings suggest an important role of HIV-1 Nef-specific CTL responses in the control of Nef activity during the progression of an HIV-1 infection.

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[3] Abbreviations used in this paper: MHC-I, MHC class I; SH3, Src homology 3; 7-AAD, 7-aminoactinomycin D; wt, wild type; IVKA, in vitro kinase assay.

Table I. *Summary of HLA-B35⁺ subjects used in this study*[a]

Pt.	HLA Class I Allele	Months since Seroconversion	Viral Load (log_{10}/ml)	CD4 (mm^{-3})	Antiretroviral Therapy	Nef Sequence	PBMC Availability
001	*A2402/A2603, B3501/B4002*	132	ND	227	+	RPQVPLRPMT**F**	−
		192	3.9	223	+	**T**PQVPLRPMTY	+
003	*A2402/A2601, B3501/B5101*	72	ND	480	−	RPQVPLRPMT**F**	−
		144	ND	252	+	**T**PQVPLRPMTY	+
006	*A24/A26, B35/B52*	48	ND	102	+	RPQVPLRPMT**F**	−
015	*A11/A24, B35/B54*	147	BD	383	+	**T**PQVPLRPMTY	+
016	*A26/A33, B35/B44*	7	ND	43	−	RPQVPLRPMT**F**	−
017	*A2/A24, B35/B48*	192	BD	254	+	**T**PQVPLRPMTY	−
019	*A2402/−, B3501/B5201*	18	4.7	524	−	RPQVPLRPMT**F**	−
		80	BD	1574	+	**T**PQVPLRPMTY	+
025	*A24/A31, B35*	26	ND	50	+	**T**PQVPLRPMTY	−
027	*A24/A26, B35/B44*	4	ND	84	+	RPQVPLRPMT**F**	−
033	*A0207/A3101, B3501/B4601*	72	5.3	326	−	**T**PQVPLRPMTY	+
034	*A2402/A2601, B3501/B4801*	48	4.4	201	−	**T**PQVPLRPMTY	+
042	*A24/A31, B35/B60*	59	3.8	311	−	**T**PQVPLRPMTY	+
046	*A2, B35/B61*	48	BD	263	+	**T**PQVPLRPMTY	+
099	*A2402/−, B3501/B61*	12	3.9	984	−	RPQVPLRPMT**F**	+
100	*A2601/−, B3501/B4001*	16	5.0	614	−	RPQVPLRPMT**F**	+
102	*A2402/A0206, B3501/B0702*	17	2.8	482	−	RPQVPLRPMT**F**	+
131	*A2402/A0207, B3501/B4601*	10	1.9	563	+	RPQVPLRPMT**F**	+
136	*A2402/A2601, B3501/B5201*	15	4.4	308	−	RPQVPLRPMT**F**	+
141	*A0201/A3101, B3501/B5401*	10	5.3	382	−	RPQVPLRPMTY	+
		20	5.1	360	+	RPQVPLRPMT**F**	+
145	*A0207/A2601, B3501/B5101*	6	BD	645	−	RPQVPLRPMTY	−
		18	4.6	685	−	RPQVPLRPMT**F**	+
161	*A2402/A2601, B3501/B5401*	13	2.3	955	−	RPQVPLRPMT**F**	+
168	*A2601/−, B3501/−*	5	2.3	408	+	RPQVPLRPMTY	+
178	*A2601/A3101, B3501/B4601*	8	2.7	568	+	RPQVPLRPMTY	+

[a] ND, Not determined; BD, below detection limit. Bold, underlined letters in the sequences represent mutations.

Materials and Methods

Subjects

A total of 23 individuals (HLA-B35⁺) with HIV infection followed at the AIDS Clinical Center (International Medical Center of Japan) were enrolled for functional analysis of HIV-specific CD8 T cells and autologous HIV-1 sequence analysis in this study. Subjects were selected based on the availability of plasma and PBMC samples as well as HLA-B*35 expression. Clinical data of all subjects are listed in Table I. Patients 01, 03, and 17 are hemophiliacs who had been infected with HIV-1 through contaminated blood products. Because the time of HIV-1 infection or the time of seroconversion was not known for these subjects, we suspect that their infection occurred in 1983 based on a survey done on Japanese hemophiliacs. In addition, 41 individuals (negative for HLA-B*35) with HIV infections were enrolled for autologous HIV-1 sequence analysis. The study was conducted in accordance with the human experimentation guidelines of the International Medical Center of Japan and Kumamoto University.

Sequence analysis of autologous HIV-1

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 30 min) of patients' plasma, after which the viral RNA was extracted from them. DNA fragments encoding Nef proteins were amplified by a nested PCR, gel purified, and sequenced directly as described (29). The fragments were cloned into a plasmid and then sequenced for phylogenetic tree analysis.

For phylogenetic tree analysis of intrapatient evolution of the *nef* gene (HXB2 coordinate, 8932–9555), nucleotide sequences were initially aligned by using Clustal W and then manually adjusted to maximize alignment of codon triplet as needed. Regions that could not be unambiguously aligned were removed from subsequent phylogenetic analysis. The MEGA3 package of sequence analysis programs was used for detailed phylogenetic analysis (30). Pairwise evolutionary distances were calculated by using the Kimura 2-parameter model for estimation of distances, and phylogenetic trees were constructed by the neighbor-joining method.

Generation of T cell clones

CTL clones or lines were established by stimulation of PBMC with a synthetic peptide, as previously described (31). Briefly, a bulk CTL culture was seeded at a density of 0.8 or 5 cells/well with a cloning mixture (irradiated allogeneic PBMC and C1R-B3501 cells pulsed with 1 μM peptide in RPMI 1640 with 10% FCS and 100 U/ml rIL-2). Two weeks later, cells showing substantial Ag-specific cytolytic activity were maintained in the medium with peptide stimulation weekly.

Preparation of HIV-1 variants

The full-length HIV-1 pNL43 derivatives in which the *nef* gene was completely removed (pNL43ΔNef) or replaced with SF2 *nef* (pNL43SF2Nef) were created earlier (32). The Arg^{75} to Thr and Tyr^{85} to Phe mutations were achieved by site-directed mutagenesis based on SF2 *nef*. 293T cells were transfected with each of the constructs, and the infectious HIV-1 virions released into the medium were collected 48 h later. The p24 Ag concentrations of virus stocks were determined by p24 Ag ELISA.

Flow cytometric analysis

HLA stabilization assay. Peptide-binding activity for HLA-B*3501 was assessed by an HLA stabilization assay using RMA-S cells expressing HLA-B*3501 as described earlier (31).

HLA tetramer analysis. The HLA-B3501 tetramers in complex with the VY8 and RY11 peptides were prepared as previously described (31). Cryopreserved PBMC of HIV-positive (2×10^6) or -negative donors (3×10^6) were stained with the PE- and allophycocyanin-labeled tetramers at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences/BD Pharmingen) and anti-CD3-FITC (DakoCytomation) at 4°C for 15 min. The $CD3^+CD8^+$ cells were gated and then analyzed for binding with the tetramers by flow cytometry (FACSCalibur; BD Biosciences).

Intracellular cytokine staining assay. Intracellular cytokine staining of Ag-specific CTL clones was done as previously described (33). Briefly, CTL clones (4×10^4 cells) were incubated with C1R-B3501 cells (4×10^4 cells) alone or pulsed with various concentrations of peptides for 6 h at 37 °C in the presence of brefeldin A (10 μg/ml). The cells were stained first with anti-CD8 mAb and 7-aminoactinomycin D (7-AAD), permeabilized in a detergent buffer, and then stained with mAb specific for IFN-γ or TNF-α (BD Biosciences/BD Pharmingen).

Cytotoxic assays

Toward peptide-loaded cells. The cytotoxic activity of the CTL clones was determined by a standard ^{51}Cr-release assay as described previously (31).

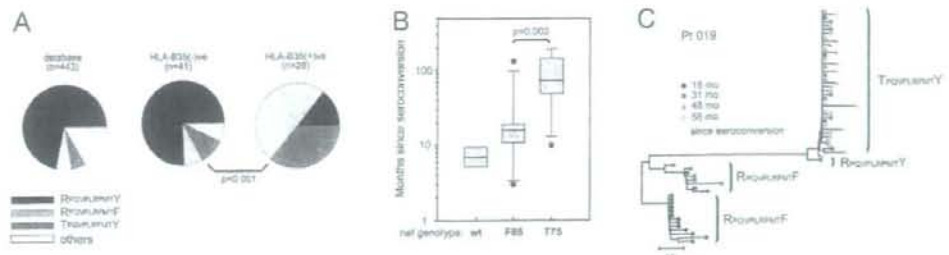

FIGURE 1. Dynamic evolution of autologous Nef sequences in HIV-infected individuals expressing HLA-B*35. *A*, Frequency of clones representing the HIV-1 Nef amino acid sequence at the RY11 epitope region as indicated in pie charts, based on the results from the Los Alamos database (*left*). The frequencies of individuals whose autologous viruses had the Nef amino acid sequences indicated when the plasma samples were collected from HIV-infected individuals negative (*middle*) or positive (*right*) for HLA-B*35 are shown. Statistical analysis was performed by using the χ^2 test. *B*, Differences in the duration of HIV infection (months since seroconversion) and the autologous *nef* genotypes, wt, Tyr^{85}Phe (F85) or Arg^{75}Thr (T75) in HLA-B35$^+$ patients. Boxes indicate values between 25th and 75th percentiles. Horizontal lines across boxes indicate the median value ± SD. Lines extend from the box to the highest and lowest values. Data include outliers (●). Statistical analysis was performed by use of the Mann-Whitney *U* test. *C*, A neighbor-joining phylogenetic tree analysis of intrahost evolution of autologous *nef* gene. Plasma HIV-1 RNA samples were collected from patient 19 at the indicated time points. The *nef* gene segment was PCR-amplified, cloned into a plasmid, and sequenced (n = 61). The amino acid sequences of the epitopic region are indicated at the *right* of the tree.

Toward HIV-infected primary CD4$^+$ cells. CD4$^+$ cells were purified from PBMC taken freshly from HIV-negative donors expressing HLA-B*3501 by using a magnetic cell separation system (Miltenyi Biotec) and stimulated with PHA (3 μg/ml; Sigma-Aldrich) for 4 days. The activated CD4$^+$ cells were then infected at relatively high titers (1 μg of p24 Ag per 10^6 cells) with wild-type (wt) or various variant HIV-1 for 6 h, and incubated for an additional 3–5 days. The HIV-infected CD4$^+$ cells (4000 cells/well) were then mixed with CTL clones at various ET ratios for 6 h at 37°C after having been labeled with ^{51}Cr. It should be noted that 30 ± 5% of the cells were p24 Ag$^+$ as revealed by intracellular flow cytometric analysis of HIV-infected CD4$^+$ cells.

HIV-1 replication assay

PBMC samples freshly isolated from HIV-seronegative donors were first infected with wt or various variant HIV-1s at 5 ng of p24 Ag in 5×10^5 cells for 4 h. The cells were washed, suspended in a culture medium (RPMI 1640, 10% FCS), and seeded in a 96-well plate at 10^5 cells/well. Three days later, the cells were stimulated with PHA at 2 μg/ml. Culture supernatants were collected and replaced with a fresh medium supplemented with human rIL-2 every 3 days. To monitor viral replication, we determined the amount of p24 Ag in the culture supernatant by a specific ELISA.

In vitro kinase assay (IVKA)

IVKA was performed as described earlier (34). Briefly, Jurkat cells (10^7) expressing wt or various variant Nef-GFP fusion proteins were lysed in KEB (50 mM Tris-HCl (pH 8), containing 137 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, Na_3VO_4, protease inhibitor mixture) at 24 h postelectroporation. Cleared lysates were immunoprecipitated with anti-GFP polyclonal Ab and the immunoprecipitates were resuspended in KAB (50 mM HEPES (pH 8), containing 150 mM NaCl, 5 mM EDTA, 0.02% Triton X-100, 10 mM $MgCl_2$) with 10 μCi of [γ-^{32}P]ATP (Amersham) for 5 min. Bound proteins were then separated by SDS-PAGE and subjected to phosphorimager (Bio-Rad) visualization and quantification. Levels of immunoisolated Nef-GFP were determined by Western-blotting of the IVKA reactions and subsequent quantification by LICOR Odyssee.

Statistical analysis

Statistical analysis and graphical presentations were done by using a computer program, SigmaPlot, with a statistical package (Hulinks). Unless otherwise indicated, results were given as median or mean ± SD. Statistical analysis of significance (p values) was based on the χ^2, Mann-Whitney rank sum, or two-tailed t test, or a one-way ANOVA, where applicable, and $p < 0.05$ was considered to be significant.

Results

*Evolution of PxxP region of Nef associated with HLA-B*35*

We previously reported that the Arg^{75} to Thr mutation (T75, amino acid numbers based on SF2 strain) in Nef was functionally associated with escape from a CTL response specific for the RY11 epitope (Nef_{75-85}: RPQVPLRPMTY) presented by HLA-B*35 in patients in the chronic phase of an HIV-1 infection (29). When we recruited more subjects including some in the early phase of infection and analyzed their autologous *nef* genotypes, another mutation, Tyr^{85} to Phe (F85), was also found in some of these HLA-B35$^+$ patients (Table I). As a result, ~50 and 40% of autologous *nef* alleles encoded the F85 and T75 mutation, respectively, in patients with HLA-B*35 (Fig. 1*A*), whereas either mutation was found in only ~5% of patients negative for HLA-B*35 as well as in all sequences from the Los Alamos HIV database (www.hiv.lanl.gov/). These data demonstrate that both T75 and F85 single mutations in Nef were differently associated with autologous viruses in patients with HLA-B*35 expression.

Because the F85 mutation was seemingly found in HLA-B35$^+$ HIV-infected subjects <2-year since seroconversion, we next analyzed the correlations between the duration of HIV infection and autologous *nef* genotypes in HLA-B35$^+$ subjects (Fig. 1*B*). The median (±SD) number of months since seroconversion in subjects with autologous wt, F85, and T75 Nef sequences was 7.0 ± 1.1, 16.0 ± 9.4, and 72.0 ± 19.5, respectively (Fig. 1*B*). This cross-sectional analysis demonstrated that HIV-1 acquired the F85 mutation earlier and the T75 mutation later concomitant with the reversion of the F85 mutation to the wt during an HIV-1 infection in subjects with HLA-B*35 expression.

*Intrahost evolution of Nef mutations associated with HLA-B*35*

To ask whether these mutations and reversions occurred sequentially within a subject, we collected plasma viral RNA samples at additional time points from three subjects, patients 001, 003, and 019. The amino acid sequence in the epitopic region sequentially changed from RPQVPLRPMTF to TPQVPLRPMTY (different amino acid residues are underlined; referred to as RF and TY, respectively, hereafter), within each subject (Table I).

To further characterize the intrapatient evolution in this region, the *nef* genotypes of plasma HIV-1 RNA of patient 19 were determined at several time points. The neighbor-joining phylogenetic tree showed that successive fixation of advantageous mutations and the extinction of unfavorable lineages had occurred, suggesting that the focus of the CTL response and/or the balance between the selective pressures that were at work on the epitope had changed over time (Fig. 1*C*). It is of interest to note that when