These macrophages were distributed around the vessels, and Hrk mRNA expression was also demonstrated in macrophages with the same pattern of distribution. These results indicate that Hrk was not a phagocytosed substance but a product of the macrophages. In addition, these perivascular macrophages constituted a specific group that was co-infected with *C. neoformans* and HIV-1.

In the CNS, the perivascular space is continuous with the subarachnoid space and provides a site for antigen presentation from macrophages to lymphocytes. Perivascular macrophages are derived from bone marrow and have the same antigens as peripheral macrophages, and they easily induce class II MHC antigens [5]. In HIVE, MGCs, which are the morphological hallmark of HIV-1 infection, and HIV-1-infected perivascular macrophages were plentiful in perivascular spaces, especially in the basal ganglia and white matter [12]. Therefore, these results suggest that HIV-1-infected peripheral monocytes penetrate the small vessels and result in the neuroinvasion of HIV-1 that causes encephalitis.

Immune response in *C. neoformans* infection is classified as a delayed hypersensitivity. In the pathogenesis of delayed hypersensitivity, apoptosis also plays important roles. Macrophages infected with some microorganisms are known to be eliminated by apoptosis to resolve the inflammation. For example, apoptosis was demonstrated in cultured alveolar macrophages from AIDS patients with disseminated pulmonary tuberculosis [16] and cultured macrophages with *Mycobacterium avium* infection [3]. In the immune response in *C. neoformans* infection, CD4+ lymphocytes play a major role in activating infected macrophages to kill intracellular bacilli [2,3,5,7–10,15–18]. Experimental studies have demonstrated that macrophage activation is required to eliminate *C. neoformans* infection in murine macrophages [2,3,5,7,9,16,17], and experimental CD4+ depletion resulted in failure to clear *C. neoformans* in pulmonary infection of rats [6]. Within un-activated macrophages, *C. neoformans* were protected from other phagocytes and continued to replicate. After the activation, TUNEL-positive cells were also observed in the *C. neoformans*-infected macrophages in liver [11] and meningitis lesions [4] in rats.

Krajewski et al. reported that the expression of the pro-apoptotic member of the bcl-family, Bax, was prominent in the perivascular microglia/macrophages in HIVE samples [10]. According to their description, productive HIV-1 infection renders macrophages more vulnerable to apoptosis, which is consistent with the limitation of microglial proliferation and activation, and with the spread of productive viral infection in the CNS with HIVE [10]. In fact, Olsen reported that bcl-2 blocked influenza virus-induced apoptosis and reduced the level of infection, virus production, and spread of the virus [14].

In the immunosuppressive state of AIDS, one cannot expect the contribution of CD4+ T cells in macrophage activation. Macrophages, charging foreign bodies for elimination, express Fas, and CD4+ T cells expressing FasL induce apoptosis [1]. Our present study suggests that when macrophages phagocytose cryptococci but fail to eliminate them, Hrk might be involved in elimination of the HIV- and *C. neoformans*-infected macrophages. Further, Hrk expression may depend on the processing stage of macrophages. Considering that some viral proteins have anti-apoptotic effects, for example CMV produces immediate-early genes, IE-1 and -2, that inhibit apoptosis, multiple pro-apoptotic factors may be required to eliminate infected macrophages. Taken together, expression of the pro-apoptotic BH3 peptide Hrk may contribute to the immune reaction against *C. neoformans* infection under the immunosuppressive state of HIV-1 infection.

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Peptide-Loaded Dendritic-Cell Vaccination Followed by Treatment Interruption for Chronic HIV-1 Infection: A Phase 1 Trial

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Immune response enhanced by therapeutic HIV-1 vaccine may control viral proliferation after discontinuation of highly active antiretroviral therapy (HAART). Although which strategies for therapeutic vaccination are feasible remains controversial, application of dendritic cells (DCs) as a vaccine adjuvant represents a promising approach to improving deteriorated immune function in HIV-1-infected individuals. The safety and efficacy of DC-based vaccine loaded with HIV-1-derived cytotoxic T lymphocytes (CTL) peptides were thus investigated in this study. Autologous DCs loaded with seven CTL peptides with HLA-A*2402 restriction were immunized to four HIV-1-infected individuals under HAART. In terms of safety, peptide-loaded DCs were well tolerated, and only mild local and general symptoms were observed during vaccine administration. ELISPOT assays to detect IFN-γ production in $CD8^+$ lymphocytes revealed a limited breadth of responses to immunized peptides in two of four participants, but no response in the remaining two participants. Differences in immunological response might be attributable to the fact that responders displayed higher nadir CD4 counts before starting HAART and were immunized with a larger number of DCs per reactive peptide than non-responders. Discontinuation of HAART after vaccination failed to lower viral set points compared to those before starting HAART. This early outcome warrants further exploration to elucidate the therapeutic value of vaccination with DCs in HIV-1 infection. ***J. Med. Virol. 78: 711–718, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: HIV-1; vaccine; HAART; treatment interruption

INTRODUCTION

Although highly active antiretroviral therapy (HAART) has significantly improved prognosis for HIV-1 infection, life-long therapy remains a requirement for continuous viral suppression [Ramratnam et al., 2000; Siliciano et al., 2003]. Long-term toxicity of HAART is therefore of medical concern and the economic cost of HAART has become a major social problem. These issues have facilitated attempts at strategic or structured treatment interruption (STI). However, successful results have not been obtained in patients starting HAART in the chronic phases, as HIV-1-specific immunity is already exhausted at the moment of treatment interruption [Oxenius et al., 2002; Fagard et al., 2003; Kaufmann et al., 2004].

Several lines of evidence have revealed that cytotoxic T lymphocytes (CTLs) play a critical role in control of HIV-1 proliferation, and that maintenance of CTL function during chronic infection requires the presence of $CD4^+$ helper T cells [Borrow et al., 1994; Koup et al., 1994; McMichael and Rowland-Jones, 2001]. However, HIV-1 selectively infects and destroys HIV-1-specific $CD4^+$ T cells, and causes quantitative and qualitative

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TABLE I. Baseline Subject Characteristics

	Subject			
	1	2	3	4
Age (years)	39	46	52	40
Sex	M	M	M	M
Duration of known seropositivity (months)	66	39	46	67
Duration of HAART (months)	65	37	42	64
HAART menu[a]	AZT + 3TC + EFV	AZT + 3TC + EFV	AZT + 3TC + EFV	d4T + 3TC + NFV
CD4 counts (/μl)				
Before HAART	164	216	50	2
At enrollment	453	658	330	340
Viral load (copies/ml)				
At enrollment	<50	<50	<50	<50
Preparation of vaccine				
Number of PBMCs obtained by leukopheresis	7.6×10^9	9.4×10^9	6.8×10^9	9.7×10^9
Number of DCs used for each vaccination	$0.7–1.2 \times 10^7$	$0.9–1.4 \times 10^7$	$1.0–1.5 \times 10^7$	$1.2–1.8 \times 10^7$
Amino acid sequences of epitope portions[b]				
Gag28	3R	3R, 7L[c]	3R	3R, 7V[c]
Gag296	wt	wt	wt	wt
Nef138	2F	5C[c]	2F	2F
Env584	4G[c]	4Q	4K, 7R, 11L[c]	wt and 4K[c]

[a]AZT, azidothymidine; 3TC, lamivudine; EFV, efavirenz; NFV, nelfinavir.
[b]wt (wild-type) represents amino acid sequences are identical to those of SF-2. Others represent amino acid positions of substitution and the substituted amino acids (see Table II).
[c]These substitutions were not included in peptides used in this study.

impairment of HIV-1-specific immunity, as shown by us and other groups [Watanabe et al., 2001; Kawamura et al., 2003]. When HAART is started and viral proliferation is controlled, destruction of $CD4^+$ T cells stops and naive lymphocytes are provided from the thymus. The immune system, however, is unable to produce and maintain HIV-1-specific immunity due to a loss of antigen stimuli under HAART. Treatment interruption in patients who start HAART in the chronic phase thus results in unfavorable outcomes.

Given this pathogenesis of HIV-1 infection, a therapeutic HIV-1 vaccine that is administered during HAART and potentiates HIV-1-specific immunity would theoretically offer a feasible strategy for achieving better viral control after STI. To test this hypothesis, we conducted a phase 1 clinical trial in which autologous dendritic cells (DCs) loaded with HIV-1-derived CTL epitope peptides were administered to four HIV-1-infected individuals and HAART was discontinued thereafter. DCs were used as highly specialized antigen-presenting cells that not only restore qualitative impairment of CTLs, but also stimulate naive $CD8^+$ T cells newly provided from the thymus during HAART [Banchereau et al., 2000]. We report herein the safety and efficacy of this DC-based therapeutic vaccine in addition to clinical outcomes after interruption of HAART.

MATERIALS AND METHODS

Participants

Subjects comprised four men with chronic HIV-1 infection and HLA genotype A*2402. All participants were under HAART with undetectable viral loads (VL; <50 copies/ml) for ≥1 year before enrollment. The institutional ethics committee approved this clinical trial and all participants provided written informed consent. Baseline characteristics are summarized in Table I.

Synthetic Peptides

Clinical-grade synthetic peptides (Table II) used for vaccination and ELISPOT assay were purchased from Multiple Peptide Systems (San Diego, CA). Gag(1–115) comprises a pool of 12- to 17-mer peptides with 10 amino acid overlaps that cover the whole Gag protein (subtype B consensus sequence) but do not include peptides containing Gag28 and Gag296 epitopes. Gag overlapping peptides were purchased from Operon Biotechnologies (Huntsville, AL). CMV-pp65 [Kuzushima et al., 2001] and EBV-TL9 [Lee et al., 1997] are both HLA-A24-restricted epitopes derived from Cytomegalovirus and Epstein-Barr virus and were purchased from Sigma-Genosys Japan (Ishikari, Japan).

TABLE II. A*2402-restricted CTL Epitope Peptides Used in This Study

Protein	Epitope	Amino acid position	Peptides used in this study	
			Designation	Sequence
Gag	Gag28	28–36	Gag28-wt	KYKLKHIVW
			Gag28-3R	KYRLKHIVW
	Gag296	296–306	Gag296	RDYVDRFYKTL
Nef	Nef138	138–147	Nef138-wt	RYPLTFGWCF
			Nef138-2F	RFPLTFGWCF
Env	Env584	584–594	Env584-wt	RYLRDQQLLGI
			Env584-4Q	RYLQDQQLLGI

RNA Extraction, PCR Amplification, and Sequencing

Viral RNA was extracted from plasma and subjected to first and second polymerase chain reaction (PCR), as described previously [Furutsuki et al., 2004]. PCR primers for Nef and Env epitope portions have been described previously [Furutsuki et al., 2004], and other primers are listed below (all nucleotide positions are in accordance with the HIV-1 SF2 strain).

For Gag28 epitope, 1st PCR primer set: forward: 5′-CGCAGGACTCGGCTTGCTGAAG-3′ (691-712) reverse: 5′-GCTATGTCACTTCCCCTTGGTTC-3′ (1506-1484). For Gag28 epitope, 2nd PCR primer set: forward: 5′-GAGAGAGATGGGTGCGAGAGC-3′ (784-804) reverse: 5′-TCTCTAAAGCTTCCTTGGTGTC-3′ (1097-1076). For Gag296 epitope, 1st PCR primer set: forward: 5′-AAGTAATACCCATGTTTTCAG-3′ (1296-1316) reverse: 5′-CTAAAATTGCCTCTCTGCATC-3′ (1947-1927). For Gag296 epitope, 2nd PCR primer set: forward: 5′-CCAGATGAGAGAACCAAGG-3′ (1474-1492) reverse: 5′-ATCTGGGTTTGCATTTTGG-3′ (1783-1765). For reverse transcriptase region, 1st primer set: forward: 5′-ATGATAGGGGGAATTGGAGGTTT-3′ (2393-2415) reverse: 5′-TACTTCTGTTAGTGCTTTGGTTCC-3′ (3422-3399). For reverse transcriptase region, 2nd primer set #1: forward: 5′-GACCTACACCTGTCAACATAATTGG-3′ (2492-2516) reverse: 5′-TAATCCCTGCATAAATCTGACTTGC-3′ (3379-3355). For reverse transcriptase region, 2nd primer set #2: forward: 5′-GTACTTTAAATTTCCCCATTAGTCC-3′ (2543-2567) reverse: 5′-CAGTCCAGCTGTCTTTTTCTGGC-3′ (3316-3294). For protease region, 1st primer set: forward: 5′-AGACAGGYTAATTTTTTAGGGA-3′ (2074-2095) reverse: 5′-TATGGATTTTCAGGCCCAATTTTTGA-3′ (2716-2691). For protease region, 2nd primer set: forward: 5′-AGAGCCAACAGCCCCACCAG-3′ (2155-2174) reverse: 5′-ACTTTTGGGCCATCCATTCC-3′ (2618-2599).

Purified PCR products were either directly sequenced or subcloned into pGEM-T vectors (Promega, Madison, WI) and sequenced using an ABI Prism dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) on a Perkin-Elmer ABI-377 sequencer.

Preparation of DCs

Leukocytes fractions were collected from participants by leukopheresis of blood samples, and peripheral blood mononuclear cells (PBMCs) were purified through a ficoll-hypaque gradient. Obtained PBMCs were aliquoted into cryotubes, and stored at −150°C until use. For induction of immature DCs, frozen PBMCs were thawed, suspended in PBS and incubated in plastic dishes for 30 min at 37°C. Adherent cells were then cultured in RPMI medium (HyClone, Logan, UT) containing 10% human AB serum (COSMO BIO, Tokyo, Japan), 50 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ), and 50 ng/ml of recombinant human interleukin 4 (PeproTech). After 6–7 days of culture, TNF-α (PeproTech) (50 ng/ml) and 7 CTL epitope peptides (10 μM each) were added and peptide-loaded mature DCs were harvested the next day. Cells were resuspended in 1 ml of saline and kept on ice until inoculation into participants. All procedures were conducted in a dedicated facility based on GCP as defined by the Japanese Ministry of Health, Labor, and Welfare.

Vaccination and Interruption of HAART

DCs loaded with HIV-1-specific epitope peptides were injected subcutaneously into axillary areas six times every 2 weeks. After the 6th vaccination, HAART was discontinued and clinical, immunological, and virological consequences were observed every week. If the HAART regimen contained efavirenz, nevirapine, or lamivudine, these antiretroviral agents were changed at least 2 weeks before treatment interruption to other agents with shorter half-lives. HAART was restarted when participants met any of the following criteria: VL > 50,000 copies/ml; VL > 5,000 copies/ml on three consecutive measurements; or CD4 counts <200/μl on two consecutive measurements.

ELISPOT Assay

For ELISPOT assay, PBMCs were aliquoted to 96-well multiscreen plates precoated with 5 μg/ml anti-IFN-γ monoclonal antibody (mAb) 1-D1K (Mabtech, Nacka Stand, Sweden). Peptides were added at concentrations of 10^{-6} M and incubated for 18 hr at 37°C. After washing wells, 100 μl of 1 μg/ml biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech) was added and incubated at room temperature for 90 min. After unbound mAb was removed, 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 using an alkaline phophatase conjugate substrate kit (BIO-RAD, Hercules, CA), and counted using a KS Elispot compact (Carl Zeiss, Oberkochen, Germany). Assays were conducted in triplicate and results were represented as mean numbers of spots per 10^6 PBMCs. When the number of spots was more than three-times the number in controls (PBMCs cultured without peptides), the response was considered significant.

RESULTS

Selection of CTL Epitope Peptides Derived From HIV-1

As immunogens for vaccination, we used HIV-1-derived peptides that were known to elicit strong CTL response and were restricted to HLA-A*2402 expressed in approximately 70% of the Japanese population (allele frequency of A*2402; 36.5%) [Tanaka et al., 1996]. The selected CTL epitope portions with A*2402 restriction were amino acid positions from 28 to 36 of Gag(Gag28), from 296 to 306 of Gag(Gag296), from 138 to 147 of Nef (Nef138), and from 584 to 594 of Env (Env584) (Table II). Whereas amino acid sequences in the Gag296 epitope portion have been shown to be conserved, other

three-epitope portions have been reported to display amino acid mutations [Ikeda-Moore et al., 1997, 1998; Dorrell et al., 1999; Furutsuki et al., 2004]. Thus, for the three-epitope portions, both wild-type peptides (Gag28-wt, Nef138-wt, and Env584-wt) and one of the representative mutant peptides (Gag28-3R, Nef138-2F, and Env584-4Q) were selected (Table II). Sequence analysis of HIV-1 derived from the four enrolled participants revealed that at least two of four epitope portions displayed amino acid sequences identical to immunized peptides (Table I).

DC-Based Vaccine Administration and Treatment Interruption

The four men enrolled in this study displayed undetectable VL under HAART (Table I). Leukopheresis was used to collect $6.8–9.7 \times 10^9$ PBMCs from each participant, and $0.7–1.8 \times 10^7$ mature DCs were harvested for each vaccination without contamination by pathogens or reactivation of autologous HIV-1. Peptides were either loaded to DCs by mixture (Subjects 1 and 2) or separately (Subjects 3 and 4), and peptide-loaded DCs were injected subcutaneously to areas near the axilla in two to three divided doses. During the course of six vaccinations in the four participants, subcutaneous bleeding (n = 1), erhythema at the injection site (n = 1), and general malaise (n = 1) were reported as local and generalized adverse events, all of which were non-serious and resolved without specific treatment (Table III).

Serum VLs were examined every week after treatment interruption and became positive above the detection limit of 50 copies/ml in all four participants, in weeks 3, 3, 1, and 2, respectively (Table III; Fig. 1). Subject 4 experienced fever at 38°C, myalgia, skin rash, and cervical lymph node swelling at 1 week after interruption, accompanied by mild liver dysfunction and thrombocytopenia, mimicking acute retroviral syndrome, and subsiding spontaneously within 2 weeks. All participants met criteria to restart HAART (at weeks 8, 4, 5, and 3, respectively) and VLs had been suppressed to undetectable levels by 11–30 weeks after restart of original HAART regimens. Differences between peak VL after treatment interruption and VL before start of HAART did not exceed 0.5 in $\log_{10}$ scale in all four participants (Table III). CD4 counts decreased after discontinuation of HAART in all participants to the level of approximately 200/μl (Fig. 1). After restarting HAART, CD4 counts in Subjects 2 and 4 gradually recovered, but those in Subjects 1 and 3 fluctuated at lower levels than prior to treatment interruption despite successful viral control by restarted HAART.

Immunological Analysis of Vaccines

HIV-1-specific CTL response to immunized peptides was evaluated by ELISPOT assay to detect IFN-γ-producing cells. Unseparated PBMCs were used for the assay, as preliminary experiments showed that IFN-γ production responding to both immunized peptides and control peptides was only seen in the $CD8^+$ population (data not shown). Significant responses to Nef138-wt were observed in Subjects 1 and 2, with weak responses to Nef138-2F in Subject 1 after the 5th vaccination (Fig. 2; black bars). Response in Subject 2 was

TABLE III. Clinical Outcomes of Vaccine Administration and Interruption of Antiretroviral Therapy

	Subject			
	1	2	3	4
CD4 counts (/μl)				
At 1st vaccination	512	310	520	428
Nadir after treatment interruption	257	252	181	213
Decrease in CD4 counts[a]	−255	−58	−339	−215
Viral load ($\log_{10}$ [copies/ml])				
Before start of HAART	4.08	5.15	4.15	5.23
Peak after treatment interruption	4.00	5.04	4.58	5.26
Reduction of VLs[b]	0.08	0.11	−0.43	−0.03
First detectable VLs after interruption (weeks)	3	3	1	2
Duration of interruption (weeks)	10	18	6	5
Adverse events				
During vaccination	Subcutaneous bleeding at injection site		General malaise	Erythema at injection site
After interruption of HAART				Fever, lymph node swelling, thrombocytopenia, elevated liver enzyme
HAART during 2 weeks before interruption	AZT + ddC + NFV	AZT + ddC + NFV	AZT + ABC + NFV	d4T + ABC + NFV
Drug-resistant mutations				
Reverse transcriptase region	None	None	None	None
Protease region	None	None	M36J (4w) none (6w)	None

[a]CD4 count at nadir after treatment interruption subtracted from count at 1st vaccination.
[b]VL before start of HAART subtracted from VL at peak after treatment interruption.

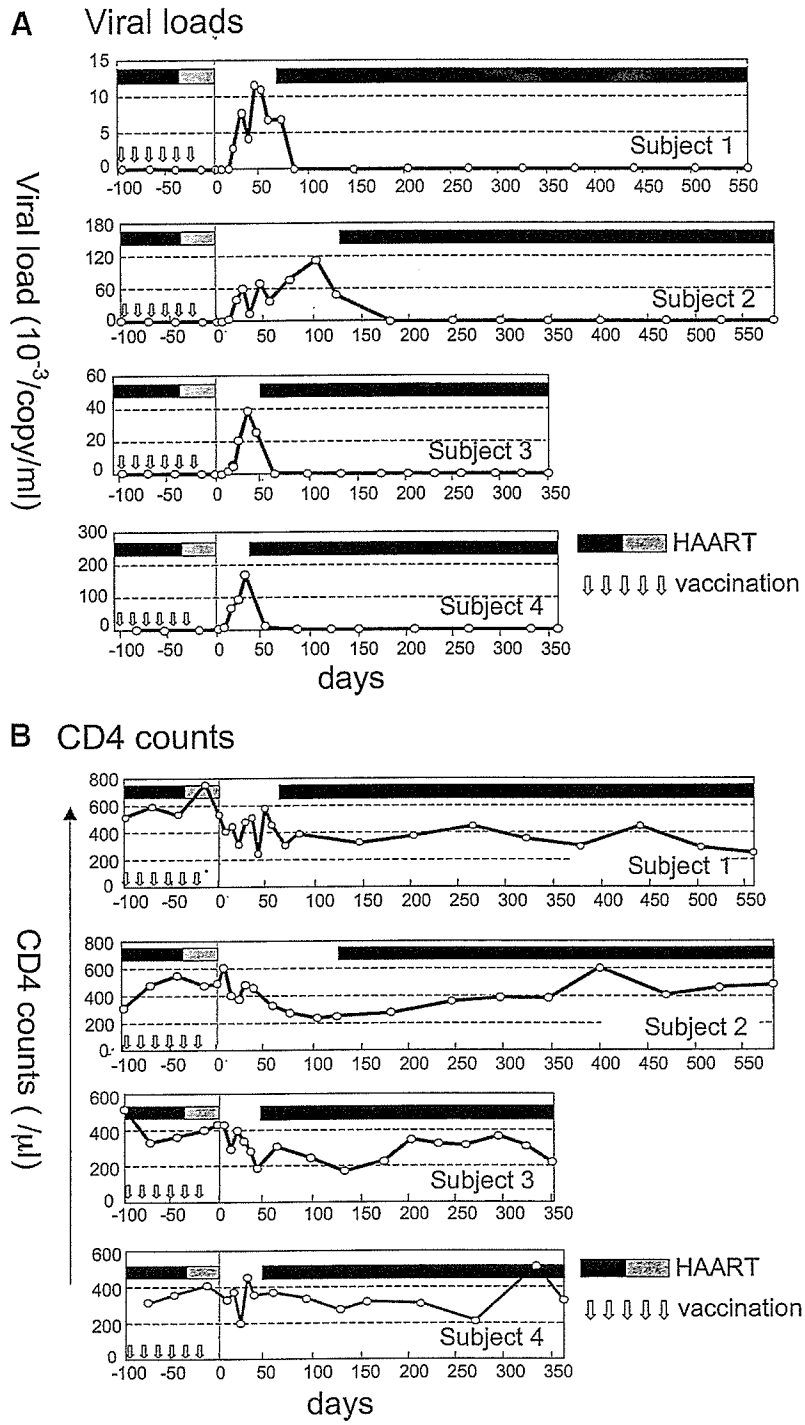

Fig. 1. Clinical courses of the four subjects. Viral loads (**A**) and CD4 counts (**B**) of the four subjects during vaccination (downward arrows) are described together with duration of treatment interruption and restart of HAART. Black bars represent duration under original HAART regimens and gray bars represent duration under alternative HAART regimens to avoid drug resistance.

specifically induced by DC-based vaccine, as no responses to control peptides of Gag(1–115), CMV-pp65, or EBV-TL9 were detected. In Subject 1, however, response was also observed to control peptides of Gag(1–115) and EBV-TL9 after the 5th vaccination, suggesting that this response to Nef138-wt and Nef138-2F included non-specific stimuli by DC injection. When HAART was discontinued and autologous virus rebounded, specific responses in Subjects 1 and 2 were induced to Nef138-wt and Nef138-2F in addition to Gag(1–115), whereas

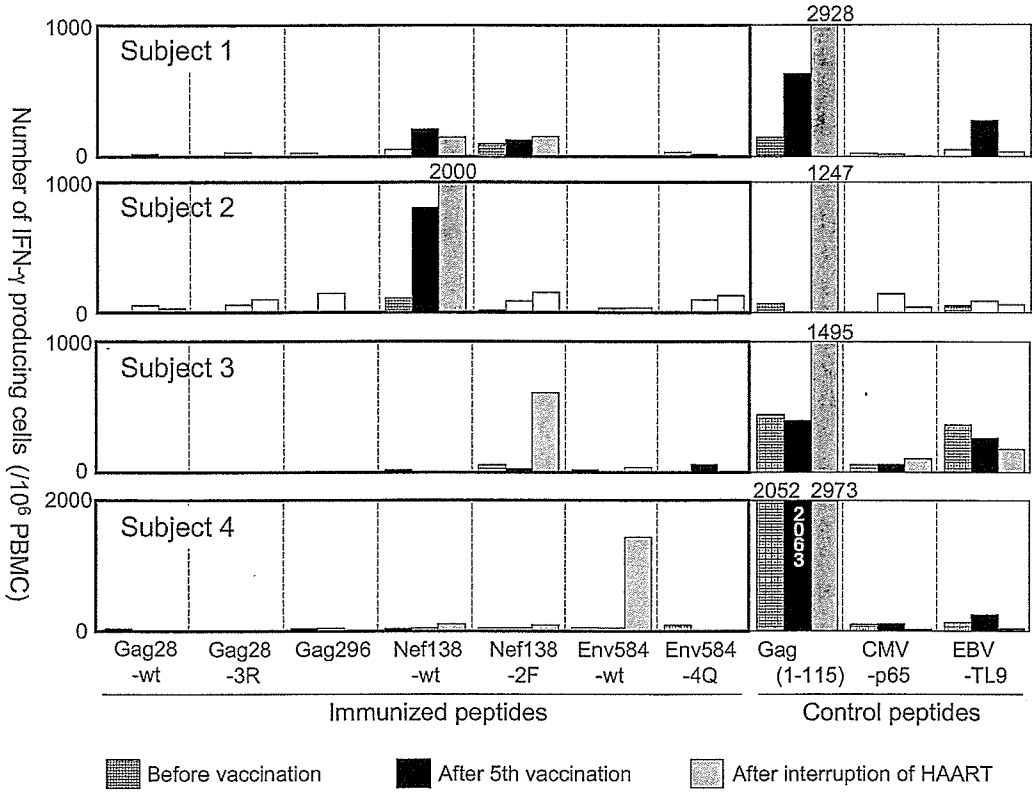

Fig. 2. Peptide-specific responses in PBMCs of vaccines. PBMCs collected before vaccination (shaded bars), after 5th vaccination (black bars) and 4 weeks after treatment interruption (gray bars), were incubated with immunized peptides; Gag28-wt, Gag28-3R, Gag296, Nef138-wt, Nef138-2F, Env584-wt, and Env584-4Q in addition to control peptides; Gag(1–115), CMV-pp65, and EBV-TL9. Response to each peptide was analyzed using ELISPOT assay detecting IFN-γ-producing cells. Specific response was calculated by subtracting number of IFN-γ-producing cells without peptides from number of cells with each peptide, and the subtracted number of cells was represented as per 10^5 PBMCs. When absolute numbers of IFN-γ-producing cells with peptides were less than three times numbers of cells without peptides, the response was considered as background and represented with white bars. To show details of data, some bars are scaled out and the specific response is represented by actual numbers of spots per 10^5 PBMCs.

no significant responses were observed for other immunized peptides.

The limited breadth of response in Subjects 1 and 2 to immunized peptides raised the possibility that differences in avidity between immunized peptides and HLA-A*2402 molecules affected the results because seven peptides were loaded by mixture into DCs for these participants. Avidity of the seven peptides was thus tested using a T2-A24 stabilization assay [Foung et al., 1986; Kuzushima et al., 2001], revealing that Env584-wt, Env584-4Q, Nef138-wt, and Nef138-2F bind HLA-A*2402 with relatively high avidity, whereas Gag28-3R binds with moderate avidity, and both Gag296 and Gag28-wt bind with low avidity (data not shown). Based on this result, each peptide for Subjects 3 and 4 was incubated with DCs separately ($\sim 1.4 \times 10^6$ DCs/peptide) and used for vaccination. However, injection of separately-loaded DCs did not induce specific response to any of the seven immunized peptides, despite the fact that rebound of autologous HIV-1 after treatment interruption induced strong responses to Nef138-2F, Env584, and Gag(1–115). We also conducted tetramer-binding assay using Nef138-wt-tetramer and ELISPOT assay using autologous DCs as antigen-presenting cells to amplify IFN-γ production, but could not find peptide-specific population or response in Subjects 3 and 4 (data not shown).

Drug-Resistance Mutations

Since one of the concerns regarding interruption of HAART is the potential emergence of drug-resistance mutations, we sequenced reverse transcriptase and protease genes of HIV-1 derived from plasma before start of HAART and 4 weeks after treatment interruption, when VLs were detectable in all participants (7,100, 58,000, 24,000, and 100,000 copies/ml, respectively). Subject 3 displayed a nucleotide substitution at position 108 in the protease regions on population sequencing, which would result in an amino acid change from methionine to isoleucine at position 36 (Table III). Sequences of clones obtained from the PCR product revealed that five of five clones displayed the M36I mutation. HIV protease genes in the plasma of this participant were further sequenced at 6 weeks after treatment interruption (29,000 copies/ml), but no M36I mutation were identified in any of the eight clones sequenced. No nucleotide substitutions were found in protease genes from the other three participants, or in reverse transcriptase genes from all participants.

DISCUSSION

After DC-based vaccination of the four subjects, immune responses to Nef138-wt in Subjects 1 and 2, and to Nef138-2F in Subject 1 were observed, whereas no detectable responses were obtained in other peptides. The results from Subjects 1 and 2 demonstrating limited breadth of response led us to consider the possibility that differences in avidity between HLA-A*2402 molecules and each peptide caused preferential presentation of Nef138 epitopes, as seven peptides were added to DCs in mixture. In fact, T2-A24 stabilization assay revealed that Gag epitopes displayed lower avidity to HLA-A*2402 than Nef138 and Env584 peptides. Thus, in Subjects 3 and 4, the seven peptides were incubated with DCs in separate wells and mixed together before vaccination, but no significant responses were observed to any of these peptides. One explanation for this observation is that when approximately 1×10^7 DCs were divided among seven peptides (approximately 1.4×10^6 cells/peptide), the numbers of DCs was too small to provide sufficient stimuli to CTLs in vivo. Although Yu et al. [2004] reported that 1.0×10^6 autologous DCs loaded with glioma-derived peptides could elicit systemic cytotoxicity in cancer patients, the number of DCs in the present cases might have been insufficient to elicit specific response from HIV-1-infected individuals. Another explanation is that Subjects 3 and 4 displayed lower nadir CD4 counts before starting HAART (50/μl and 2/μl, respectively) than Subjects 1 and 2 (164/μl and 216/μl, respectively). In untreated HIV-1-infected individuals, $CD4^+$ T cells are continuously destroyed during all stages of HIV-1 infection, causing not only quantitative, but also qualitative abnormalities in HIV-1-specific immunity. These abnormalities are carried over even after CD4 counts are normalized by the initiation of HAART. In fact, Lange et al. [2003] reported that responses to immunization of tetanus and diphtheria toxoids in chronically HIV-1-infected patients under HAART correlate with previous nadir CD4 counts, but not with current circulating CD4 counts. This kind of impaired immune function in HIV-1-infected individuals under HAART may also explain the limited breadth of immune response in Subjects 3 and 4.

In terms of safety, peptide-loaded DCs were well tolerated, and only mild local and general symptoms were observed during vaccine administration, with only one episode of acute retroviral syndrome after STI. Since treatment interruption sometimes causes viral mutation resulting in antiretroviral drug resistance [Schweighardt et al., 2002; Metzner et al., 2003; Tremblay et al., 2003], all participants changed from antiretroviral agents that are known to be susceptible to resistance mutations to other agents with short-half lives before STI. However, an M36I mutation in a protease region was transiently detected in Subject 3 when VL rebounded 4 weeks after treatment interruption, and disappeared 6 weeks after interruption. M36I mutation is regarded as one of the minor resistance mutations that can appear after emergence of major resistance mutations. We cannot determine the mechanism underlying this transient appearance of M36I in Subject 3, but replication of mono- or oligoclonal HIV-1 from reservoir cells may be responsible.

In this study with a small number of participants, DC-based vaccine elicited a limited breadth and strength of immune response, and treatment interruption failed to control rebound of viral proliferation. Several groups have tried similar therapeutic vaccines to interrupt antiretroviral therapy in both humans [Hejdeman et al., 2003; Bostrom et al., 2004; Harrer et al., 2005; Kinloch-de Loes et al., 2005; Tubiana et al., 2005; Wu et al., 2005] and macaques [Lisziewicz et al., 2005] using recombinant proteins or genes expressing HIV-1 proteins, and have reported various results of specific immune reaction and clinical outcomes after treatment interruption. Although the question as to which strategy for therapeutic vaccination is suitable for successful treatment interruption remains controversial, application of DCs as vaccine adjuvant appears theoretically attractive to improve deteriorated immune function in HIV-1-infected individuals. In agreement with this concept, therapeutic vaccine using DCs in cancer treatment has been shown to result in better tumor regression compared to vaccines using peptide alone, viral vectors or tumor cells [Banchereau and Palucka, 2005]. Recently, two groups reported preliminary results of DC-based therapeutic vaccine in HIV-1-infected patients using autologous HIV-1 as immunogens in untreated [Lu et al., 2004] and treated patients [Garcia et al., 2005]. Garcia et al. showed that DC-based HIV-1 vaccine in patients under HAART did not elicit specific immune responses, although the vaccine suppressed viral rebound in 4 of 12 vaccines after treatment interruption. Our result thus provides encouraging evidence that DC-based vaccines can induce specific immune response, albeit insufficient to suppress viral rebound, in patients under HAART. These early outcomes warrant further exploration to establish the therapeutic value of vaccination with DCs in HIV-1 infection.

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CASE REPORT

AIDS-related cerebral toxoplasmosis with hyperintense foci on T1-weighted MR images: A case report

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Summary The neuroradiological findings are helpful for the diagnosis of toxoplasmic encephalitis. The T1 hypersignal intensity foci on brain magnetic resonance (MR) images without contrast enhancement are presented and can be a pathognomonic sign of this disease.

Introduction

Most toxoplasmic encephalitis is opportunistic infection complicated with the acquired immunodeficiency syndrome (AIDS) and immunosuppressive conditions. The diagnosis of this disease is difficult because of the incompetence of the serological examination for the immunocompromised patients.[1] Although the direct detection method for the pathogen by polymerase chain reaction (PCR) using the cerebrospinal fluid (CSF) has high specificity, the sensitivity of this method is insufficient for definitive diagnosis.[2] We, therefore, have to synthetically diagnose with clinical symptoms, signs, laboratory data, neuroradiological images and the response to anti-toxoplasmosis therapy.

We report here our experience of a unique MR imaging finding of toxoplasmic encephalitis in an

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AIDS patient and emphasize the hyperintense foci on T1-weighted MR images that can be one of the pathognomonic MR images of this disease.

Case report

A 44-year-old man with disturbance of consciousness and respiratory insufficiency was admitted to our hospital in April 2005. His consciousness had been rapidly deteriorated and he developed coma 2–3 days before hospitalization. Serological tests of HIV antibodies and *Toxoplasma gondii* IgG antibody were positive, but the *T. gondii* IgM antibody was not detected. The concentration of HIV RNA in plasma was 120,000 copies/ml and the CD 4 cell count was 8 μl. The chest X-ray showed bilateral ground glass shadow and *Pneumocystis jiroveci* (carinii) was detected from bronchoalveolar lavage (BAL) fluid. CSF showed mild elevated protein level of 65 mg/dl and pleocytosis, and the opening pressure was over 300 mmH_2O. No malignant cells or microorganisms were detected. *T. gondii* B1-gene fragment was detected by PCR using CSF, therefore, the diagnosis of an AIDS case with toxoplasmic encephalitis was made.[3]

MRI of the brain showed multiple high intensity lesions on T2-weighted image (Fig. 1a) and the corresponding T1-wighted image showed low intensity lesions. Contrast enhanced T1-weighted images showed multiple nodular and ring enhancement lesions.

The chemotherapy with trimethoprim/sulfamethoxazole (TMP/SMX) was very effective and the patient's consciousness level was improved gradually. *P. jiroveci* pneumonia was also cured. MR imaging after 4 weeks of treatment demonstrated that the multiple nodular lesions on T1 and T2-weighted images had significantly been reduced. After 8 weeks of treatment, the contrast enhanced T1-weighted images showed only residual small lesions without contrast enhancement. Interestingly, the hypersignal intensity foci appeared at bilateral basal ganglia obviously after 2 weeks of treatment on the non-enhanced T1-weighted images (Fig. 1b). Corresponding computed tomography (CT) image did not show hemorrhagic or calcified densities (Fig. 1c). These T1 hypersignal intensity foci regressed gradually along with anti-toxoplasmic chemotherapy in proportion to other mass lesions. The T2* (star)-weighted image, which can detect the hemosiderin deposition as hypointensity lesion, operated after 12 weeks of treatment showed no hypointensity at corresponding T1 hypersignal intensity foci on basal ganglia (Fig. 1d).[4] We concluded that the toxoplasmic encephalitis showed the hypersignal intensity foci on T1-weighted MR imaging without hemorrhage or calcification.

Discussion

Toxoplasmic encephalitis progresses rapidly and is life threatening to immunocompromised patients. Therefore, we often have to start the anti-toxoplasmosis therapy when this encephalitis is suspected on the neuroradiologic images and laboratory data. Typically, the toxoplasmic encephalitis lesions on MRI studies appear as T2 hypersignal intensity foci and T1 hypo-isosignal intensity foci, and reveal a rim of enhancement surrounding the edema on contrast enhanced T1-weighted images. Nevertheless, even characteristic foci on these MR images are not pathognomonic. Since the differential diagnosis of toxoplasmic encephalitis from other infections or CNS lymphoma is difficult, improvement in the diagnostic methods is an urgent necessity.

In our case, the toxoplasmic encephalitis was diagnosed with the highly specific PCR and confirmed by the response to anti-toxoplasmosis therapy. Brain MRI revealed unusual findings, T1 hypersignal intensity foci, accompanied by typical multiple high intense lesions on T2-weighted image during the treatment. These unique MR findings have been reported on only a few cases of non-HIV/AIDS-related toxoplasmic encephalitis. Terada et al.[5] reported a case of toxoplasmic encephalitis after stem cell transplantation with T1 hypersignal intensity foci. Autopsy revealed the disseminated toxoplasmosis, and coagulative necrosis without hemorrhage or calcification was revealed at corresponding T1 hypersignal intensity foci by neuropathological study. In another post-bone marrow transplantation case, inflammatory and vascular changes without hemorrhage appeared to be the cause of iso or hypersignal intensity rings by the stereotactic biopsy of T1 hypersignal intensity foci.[6] On the other hand, Navia et al.[7] demonstrated that the T1 hypersignal intensity foci were caused by coagulative necrosis with lipid-laden macrophages. The pathophysiological and neuroradiological mechanisms to create these MRI findings are far from clear yet. The reason why the T1 hypersignal intensity foci tend to localize in the basal ganglia is not clear either.[5,6]

CNS lymphoma, which is important for the distinction from toxoplasmic encephalitis, shows T1 hypo-isosignal intensity foci and never shows T1 hypersignal intensity foci except subacute

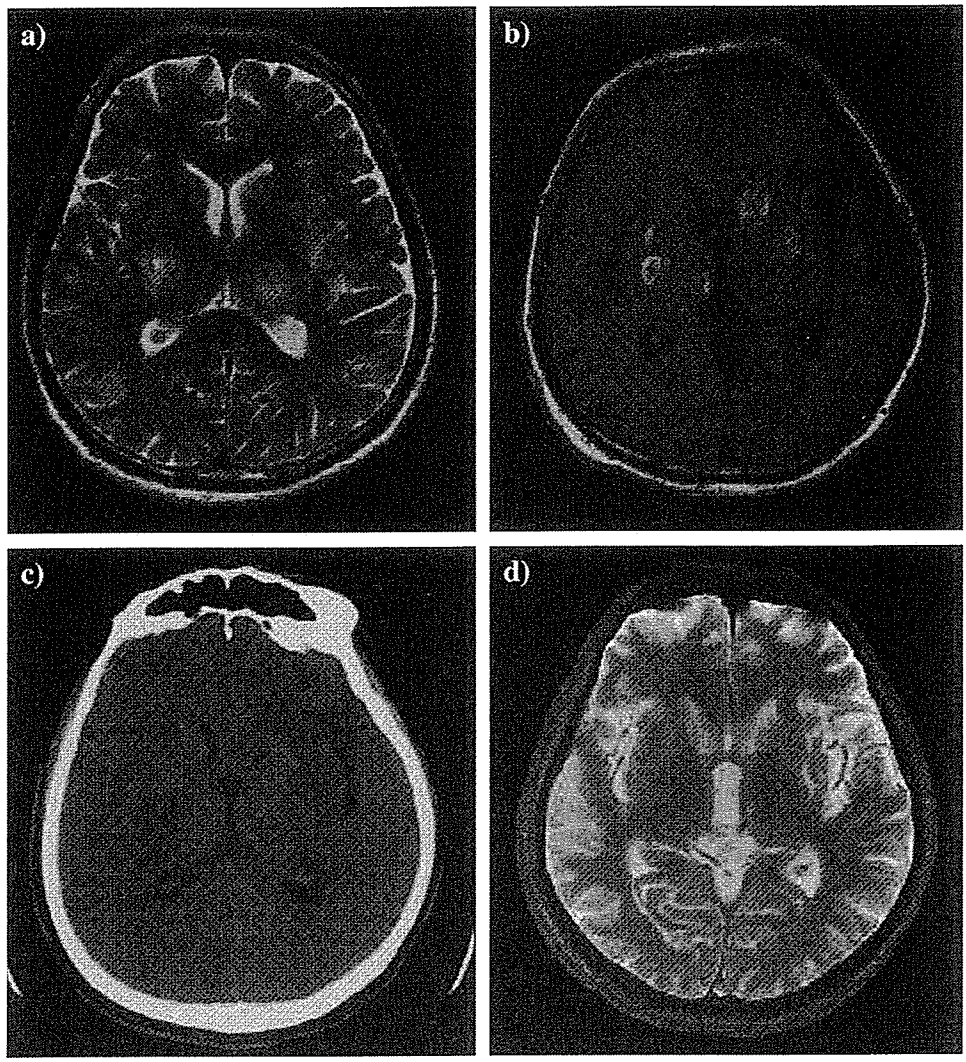

Figure 1 (a) The T2-weighted magnetic resonance image presented multiple high intense lesions. (b) Non-enhanced T1-weighted image showed hypersignal intensity foci at bilateral basal ganglia. (c) The corresponding CT image showed non-hemorrhagic or non-calcified density. (d) The T2* (star)-weighted image showed non-hemorrhagic observations at basal ganglia.

hemorrhage with hypervascular CNS lymphoma.[8,9] However, the CT imaging and T2* (star)-weighted MR imaging can simply distinguish it from the toxoplasmic T1 hypersignal intensity foci without hemorrhage or calcification.

We reported here the unique MRI findings, T1 hypersignal intensity foci, without hemorrhage or calcification on HIV/AIDS-related toxoplasmic encephalitis. It will be helpful for the diagnosis of toxoplasmic encephalitis and may be a pathognomonic finding. Unfortunately, since this report we are yet to experience another case of toxoplasmic encephalitis, but we would like to continue to explore this unique MRI findings of this disease.

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Sequence Note

Active Generation and Selection for HIV Intersubtype A/D Recombinant Forms in a Coinfected Patient in Kenya

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ABSTRACT

To investigate the *in vivo* evolution of recombinant HIV, we followed up on a mother who was initially coinfected with subtypes A and D in Kenya. Blood samples were obtained in 1996 and 2002, and HIV *pol* and *env* genes were amplified by PCR, cloned, sequenced, and phylogenetically analyzed. As for the 1996 sample most of the clones generated from the *pol* and *env* genes clustered either with subtypes A and D reference strains. However, two clones from the *pol* gene were found to be independent recombinants between subtypes A and D by RIP analysis, suggesting active generation of recombinant forms. As for the 2002 sample, all the clones from the *pol* gene clustered only with the subtype A reference strain, while all the *env* clones clustered only with subtype D, denoting a dominance of an A/D recombinant form. These results indicate that in patients dually infected with subtypes A and D there is an ongoing generation and selection for A/D recombinant forms.

THE GENOME OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) is in a rapid evolutionary state. Presently, HIV has been classified into three main groups based on their genomic sequences: M (major), and two divergent groups, O (outlier) and N (non-M, non-O).[1] The vast majority of variants found worldwide and responsible for the AIDS pandemic belong to group M. Phylogenetic analysis of group M has further led to its subdivision to pure subtypes A to K and subsubtypes A1, A2, F1, and F2.[2] Recently, it was realized that a significant fraction of isolates is of an intersubtype form composed of genomes from two or more different subtypes. These mosaics are thought to be generated by recombination arising from coinfection of the same target cell with different viruses, leading to production of heterodimeric virions.[3] They are highly likely to have arisen in geographic regions where distinct HIV subtypes cocirculate. Some of these mosaic genomes are unique and have been restricted to small clusters. However, others have spread globally infecting unlinked individuals and are now designated as circulating recombinant forms (CRF).[1,2] The contribution of recombinant viruses to the global AIDS pandemic is significant. The spread of CRF01-AE (CM240) in Thailand,[4] the dominance of CRF02-AG (CRF02) in West and Central Africa,[5] and the recent explosive outbreaks of HIV in Russia and China attributed to CRF03-AB and B/C, respectively,[6,7] suggest that HIV recombinant variants will have profound implications in anti-HIV prevention and control strategies.

In Kenya, though pure subtypes A1 and D are the most common HIV subtypes,[5] there has been a rise in reported cases of recombinants especially in the Western region.[8] Samples collected between 1997 and 1998 showed that 26.8% were recombinants,[9] while those collected 2 years later showed a more than 40% recombination.[10] Expectedly, the A/D recombinant form has been the most prevalent, accounting for more than 46% of all reported recombinant cases. The increased reporting of the A/D recombinants in the country has lead to speculation that in areas where A and D subtypes coexist, the inter-

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subtype A/D recombinant form may be preferentially selected. However, as HIV molecular studies in Kenya have mostly been cross-sectional, there has been little information on longitudinal follow-up, which has precluded analysis of their *in vivo* evolution. Demonstrating the types of HIV favored by selection mechanisms will increase our understanding of its evolution for the design of effective anti-HIV therapeutic and vaccine strategies. In this study, we report our findings on a mother in western Kenya who initially was coninfected with HIV subtypes A and D but developed the A/D recombinant form during follow-up.

The patient involved (KS004) was in 1996 a 22-year-old antenatal clinic attendee participating in a study on the prevention of HIV mother-to-child transmission using short-course zidovudine in rural western Kenya.[11] She was among a group of mothers who, due to reasons earlier outlined,[12] could not comply with zidovudine use. The initial blood sample was obtained at recruitment in August 1996 and another sample was obtained 6 years later (January 2002), on a follow-up program to determine eligibility of use of full-course antiretroviral therapy. On each occasion the sample was separated into peripheral mononuclear cells using Ficoll-Paque, and genomic DNA was extracted with DNAzol (GIBCO-BRL, Grand Island, NY). Polymerase chain reaction (PCR) amplification was performed on the viral *pol* and *env* regions. A part of the *pol* region encoding the reverse transcriptase gene (corresponding to 2480–3180 nt of HIV-1 HXB2) was amplified by nested PCR with

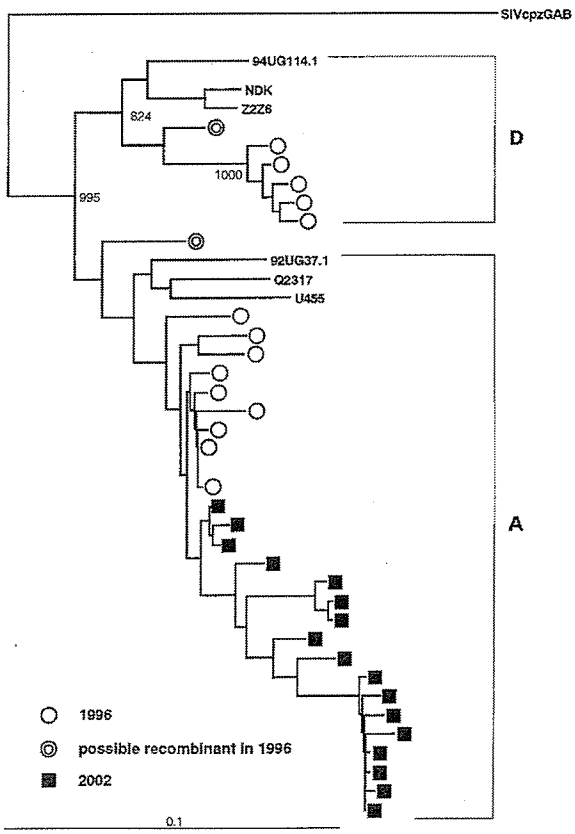

FIG. 1. Phylogenetic analysis of the *pol*-RT gene (697 bp) of HIV-1 clones isolated in 1996 (○, ◎) and 2002 (■) from an HIV-infected mother (KS004) in Kenya.

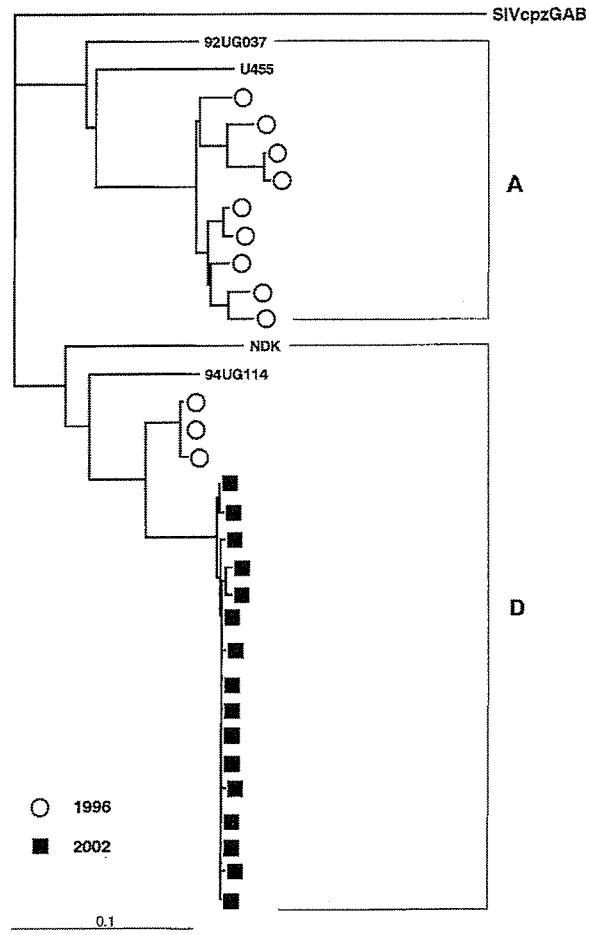

FIG. 2. Phylogenetic analysis of the *env*-C2V3 gene (550 bp) of HIV-1 clones isolated from KS004 in 1996 (○) and 2002 (■).

primer pairs RT18 (5′-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3′) and KS104 (5′-TGACTTGCCCAATTTAGTTTTCCCACTAA-3′) in the first round and KS101 (5′-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3′) and KS102 (5′-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3′) in the second round. Cycling conditions included a hot start at 95°C for 10 min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min and a final extension at 72°C for 10 min. For the amplification of part of the *env* gene (corresponding to 6954–7504 nt of HIV-1 HXB2) primer pairs M5 (5′-CCAATTCCCATACATTATTGTGCCCCAGCTGG-3′) and M10 (5′-CCAATTGTCCC TCATATCTCCTCCTCCAGG-3′) were used for the first round and primer pairs M3 (5′-GTCAGCACAGTACAATGIACACATGG-3′) and M8 (5′-TCCTTGGATGGGAGGG GCATACATTGC-3′) for the second round. Cycling conditions for both fragments were similar and included a hot start at 95°C for 10 min, then 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, and a final extension of 72°C for 10 min. The PCR products arising from four independent PCR reactions (approximately 697 bp for *pol* and 550 bp for *env*) were separately cloned by using a pCRII vector (In-

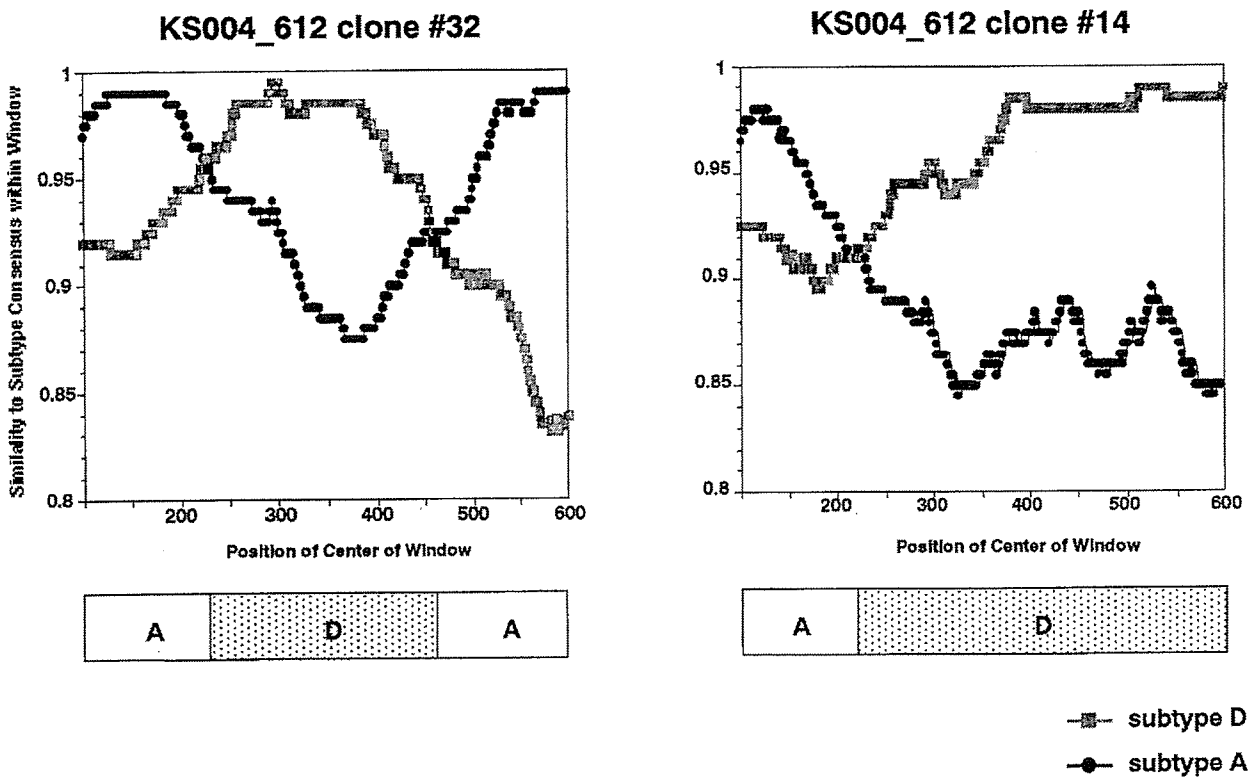

FIG. 3. RIP output and schematic representation of the mosaic structure of the *pol*-RT gene (697 bp) of HIV-1 clones (KS004_612 clones #14 and #32) isolated from KS004 in 1996.

vitrogen, Carlsbad, CA) and plasmid DNA for sequencing prepared by Quantum miniprep kit (Bio-Rad Life Sciences, Hercules, CA). DNA sequencing was carried out by dye-deoxy terminator chemistry on an ABI 310 automatic sequencer (Applied Biosystems, Foster City, CA). At least 12 plasmid clones were sequenced from each fragment and from samples collected in both of the years to obtain the consensus sequence. DNA sequences were aligned by using CLUSTAL W, version 1.81, with subsequent inspection and manual modification. Phylogenetic trees were constructed by the neighbor-joining method and its reliability was estimated by 1000 bootstrap replications. All alignments were gap stripped for the generation of trees. Resulting trees were visualized using Treeview, version 1.65. To determine recombination breakpoints, the recombinant identification (RIP) analysis was performed.[1]

The outcome of the phylogenetic analysis of the reverse transcriptase *pol* gene region (697 bp) of representative clones of both the 1996 and 2002 samples is shown in Figure 1. Clones derived from samples collected in 1996 clustered with representative sequences from either subtype A or D in both the *pol* (Fig. 1) and *env* gene (Fig. 2). Two clones generated from the *pol* gene of the 1996 sample did not cluster with any reference strains, and RIP analysis revealed that they were independent recombinant forms between subtypes A and D (Fig. 3). Clones isolated from the 2002 sample all clustered within the subtype A sequences in the *pol* and subtype D in *env* gene. These re-

AA POSITION	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	Net charge
Consensus D	C	T	R	P	Y	N	N	T	R	Q	S	T	H	I	G	P	G	Q	A	L	Y	T	T	K	.	I	I	G	D	I	R	Q	A	H	C	
KS004_612-04	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	R	-	W	-	-	-	E	.	-	V	-	-	-	-	-	-	-	+2
KS004_612-05	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	R	-	W	-	-	-	E	.	-	V	-	-	-	-	-	-	-	+2
KS004_612-10	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	R	-	W	-	-	-	E	.	-	V	-	-	-	-	-	-	-	+2
KS004_201-01	-	-	-	-	-	-	K	-	T	I	Y	S	Y	R	-	-	-	R	-	W	-	-	-	T	.	-	R	-	-	-	-	-	-	-	+6
KS004_201-02	-	-	-	-	-	-	K	-	T	I	Y	S	Y	R	-	-	-	R	-	W	-	-	-	T	.	-	R	-	-	-	-	-	-	-	+6
KS004_201-03	-	-	-	-	-	-	K	-	T	I	Y	S	Y	R	-	-	-	R	-	W	-	-	-	T	.	-	R	-	-	-	-	-	-	-	+6
KS004_201-05	-	-	-	-	-	-	K	-	T	I	Y	S	Y	R	-	-	-	R	-	W	-	-	-	T	.	-	R	-	-	-	-	-	-	-	+6

FIG. 4. Predicted amino acid changes in the V3 loop of KS004 clones isolated in December 1996 (KS004_612) and in January 2002 (KS004_201).

sults suggest that in 1996 the patient was coinfected with a likelihood of various viral variants, such as either pure subtypes A and D, a combination of the pure subtypes and recombinants, or various types of recombinants. However, by 2002 only one variant, an A/D recombinant, had been selected for dominance.

The predicted amino acid changes in the V3 loop are shown in Figure 4. The V3 loops of the subtype D clones isolated in 1996 and those isolated in 2002 had 34 amino acids, which is consistent with other subtype D clones from the region.[1] However, there was a predicted shift in coreceptor usage from CCR5 to CXCR4 based on the change in the net amino acid charge, from +2 in 1996 to +6 in 2002.[13] Similar amino acid changes were observed at position 7 and a whole peptide change, RQGTHI to TIYSYR, from positions 9 to 14.

Overall, isolates obtained from the 1996 sample showed greater diversity in the subtype A (both *pol* and *env*) than in the subtype D sequences. But those obtained in 2002 were almost a clonal line of *env* sequences with very little diversity. This homogeneity in *env* sequences from 2002 may be due to a waning of the host immune pressure, though the possibility of an artifact arising from our PCR and cloning procedures cannot be excluded. We are currently analyzing the viral RNA to compare the circulating virus with the proviral DNA to confirm these observations.

The emergence and dominance of the A/D recombinant form confirm not only that recombination *in vivo* occurs in dually infected patients, but also that particular types of recombinants may be preferred by selection processes over the parental subtypes. A recent report from the same location in Kenya has shown a preference for vertical transmission of subtype D containing *gag* and *env* fragments over subtype A.[14] Similarly, in a large study cohort in neighboring Uganda, there have been reports of faster progression to AIDS among cases infected with HIV-1 subtype D than those with subtype A in the *env* region.[15] The preferred selections for *env* D over *env* A in our study case may support the hypothesis that viruses containing subtype D sequences in the *env* region have better fitness capacity *in vivo*.

Intersubtype recombination may be a common occurrence in Kenya and plays a central role in the emergence of new HIV variants. However, for the design of effective anti-HIV strategies, there is a need for more studies in areas where multiple HIV variants cocirculate to establish why some subtypes or recombinants have an ability to subsequently "win" over the rest for dominance.

GeneBank accession numbers: deposit of samples to GeneBank in process.

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Genetic Diversity of HIV Type 1 in Rural Eastern Cameroon

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Summary: To monitor the presence of genotypic HIV-1 variants circulating in eastern Cameroon, blood samples from 57 HIV-1-infected individuals attending 3 local health centers in the bordering rural villages with Central African Republic (CAR) were collected and analyzed phylogenetically. Out of the 40 HIV-1 strains with positive polymerase chain reaction (PCR) profile for both *gag* and *env*-C2V3,12 (30.0%) had discordant subtype or CRF designation: 2 subtype B/A (*gag/env*), 1 B/CRF01, 2 B/CRF02, 1 CRF01/CRF01.A, 2 CRF11/CRF01, 1 CRF13/A, 1 CRF13/CRF01, 1 CRF13/CRF11, and 1 G/U (unclassified). Twenty-eight strains (70.0%) had concordant subtypes or CRF designation between *gag* and *env:* 27 subtype A and 1 F2. Out of the remaining 17HIV-1 strains negative for PCR with the *env*-C2V3 primers used, 10 (58.8%) had discordant subtype or CRF, and 7 (41.2%) had concordant one based on *gag/pol/env*-gp41 analysis. Altogether, a high proportion (22/57, 38.6%) of the isolates were found to be recombinant strains. In addition, an emergence of new forms of HIV-1 strains, such as subtype B/A (*gag/env*), B/CRF01 and B/CRF02, was identified. The epidemiologic pattern of HIV-1 in eastern Cameroon, relatively low and high prevalence of CRF02 and CRF11, respectively, was more closely related to those of CAR and Chad than that of other regions of Cameroon, where CRF02 is the most predominant HIV-1 strain. These findings strongly suggest that this part of Cameroon is a potential hotspot of HIV-1 recombination, with a likelihood of an active generation of new forms of HIV-1 variants, though epidemiologic significance of new HIV-1 forms is unknown.

Key Words: HIV, subtypes, CRF, recombinant, hotspot

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Recent studies have suggested that simian immunodeficiency virus of chimpanzee (SIVcpz) has been introduced into humans at least 3 times, resulting in HIV-1 groups M, N, and O.[1–4] The cross-species transmission of SIVcpz is now thought to have occurred through humans being exposed to the blood of chimpanzees infected with SIVcpz during hunting and butchering of these chimpanzees in rural areas of the equatorial rain forest of Central Africa.[4–6]

The epidemiologic pattern of HIV-1 infection in Cameroon, a west-central African country, is characterized by an extensive genetic variability of the virus in terms of cocirculation of all representative major groups and subtypes,[1–4] though CRF02_AG and subtype A represent 70–80% of HIV-1 infection in the country.[7–18] HIV-1 group O seems to be endemic in Cameroon, where the frequency is estimated to be 2–5% among HIV-1-infected individuals.[19,20] Group N, the least spreading virus group among HIV-1 isolates, is represented by only a handful of viruses identified in some villages in Cameroon. Overall seroprevalence of HIV-1 in rural villages has been reported to be relatively low (0.7–6%) compared with urban and semiurban areas (11%) in Cameroon.[5,6,21] However, most studies have been focused on patients living in major urban areas, and less information is available on the distribution of group M strains circulating in rural areas,[5] where >60% of Cameroonians reside.

In the east of Cameroon, Chad, relatively low prevalence of CRF02_AG (13.1%) and relatively high prevalence of CRF11_cpx (13.1–18.0%) and subtype D (18.7%) compared with those in neighboring countries, have been reported. In the southeast of Cameroon, the Central African Republic (CAR), a high prevalence of subtype A (69.7%) and CRF01_AE (21.8%) has been reported,[22,23] showing that the subtype distribution is heterogeneous in west-central Africa.[24,25]

The purpose of this study was to investigate the genetic diversity of HIV-1 strains circulating in rural and border villages of eastern Cameroon, to compare the result with those of Chad and CAR, and to determine the influence of migration of people across the border on the genetic diversity of HIV-1 in the eastern part of Cameroon.

MATERIALS AND METHODS

Study Population

The subjects enrolled in this study were HIV-1-infected individuals recruited in September 2001 in 3 semirural areas of eastern Cameroon near Bertoua, a town on the road connecting

the capital cities of Cameroon (Yaounde) and CAR (Bangui). They were 52 Cameroonians and 5 CAR citizens attending 3 local health centers and suspected of or having sexually transmitted infection, tuberculosis, or AIDS. After obtaining informed consent and ethical clearance, 10 mL of blood samples were collected from the 57 patients (27 men and 30 women, mean age ± SD: 31.5 ± 10.0 years) (Table 1) and screened for HIV antibodies. The plasma samples were confirmed to be positive for HIV antibodies with a microparticle enzyme immunoassay kit (AxSYM HIV1/2; Abbott Japan) and an immunochromatography assay kit (Determine HIV1/2; Abbott Japan). The peripheral blood mononuclear cells were prepared by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Genomic DNA was extracted from peripheral blood mononuclear cells of sero-reactive samples using a DNA extraction kit (Qiagen, Hilden, Germany).

Polymerase Chain Reaction, Cloning, and Sequencing

A part of HIV-1 group M *env* gene covering C2V3 (corresponding to 6975–7520 nt in HIV-1_{HXB2}) was amplified by nested polymerase chain reaction (PCR) with primers M5 (5′-CCAATTCCCATACATTATTGTGCCCCAGCTGG-3′) and M10 (5′-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3′) in the first round, and M3 (5′-GTCAGCACAGTACAATGCACACATGG-3′) and M8 (5′-TCCTTGGATGGGAGGGGCATACATTGC-3′) in the second round.[6] A part of *gag* encoding p24 (corresponding to 1596–2016 nt in HIV-1_{HXB2}) was amplified by nested PCR using primers H1G777 (5′-TCACCTAGAACTTTGAATGCATGGG-3′) and H1P202 (5′-CTAATACTGTATCATCTGCTCCTGT-3′) in the first round, and H1gag1584 (5′-AAAGATGGATAATCCTGGG-3′) and g17 (5′-TCCACATTTCCAACAGCCCTTTTT-3′) in the second round.[7] A part of *env* gene encoding gp41 (corresponding to 7880–8280 nt in HIV-1_{HXB2}) was amplified by nested PCR with primers GP40F1 (5′-TCTTAGGAGCAGCAGGAAGCACTATGGG-3′) and GP41R1 (5′-AACGACAAAGGTGAGTATCCCTGCCTAA-3′) in the first round, and GP46F2 (5′-ACAATTATTGTCTGGTATAGTGCAACAGCA-3′) and GP47R2 (5′-TTAAACCTATCAAGCCTCCTACTATCATTA-3′) in the second round.[26] A part of *pol* gene encoding integrase (IN) (corresponding to 4493–4780 nt in HIV-1_{HXB2}) was amplified by nested PCR using primers, unipol 5 (5′-TGGGTACCAGCACACAAAGGAATAGGAGGAAA-3′) and unipol 6 (5′-CCACAGCTGATCTCTGCCTTCTCTGTAATAGACC-3′) in the first round, and unipol 1 (5′-AGTGGATTCATAGAAGCAGAAGT-3′) and unipol 2 (5′-CCCCTATTCCTTCCCCTTCTTTTAAAA-3′) in the second round.[6]

Nested PCR was performed with an AmpliTaq Gold PCR kit (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions. Amplification was done with one cycle of 95°C for 10 minutes and 35 cycles of 95°C for 30 seconds, 45–55°C for 30 seconds, and 72°C for 1 minute with a final extension of 72°C for 10 minutes. Samples that could not be amplified with the *env*-C2V3 or the *gag*-p24 primer sets were analyzed with *env*-gp41 and *pol*-IN-specific primers. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel. The amplified products were cloned with TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced as previously described.[6] At least 5 clones per sample were analyzed to obtain a consensus sequence.

Genetic Distance and Phylogenetic Analysis

The sample sequences were aligned with subtype reference sequences from the Los Alamos database by CLUSTAL W (version 1.81) with subsequent inspection and manual modification. The frequency of nucleotide substitution in each base of the sequences was estimated by the Kimura 2-parameter method.[27] A phylogenetic tree was constructed by the neighbor-joining method,[28] and its reliability was estimated by 1000 bootstrap replications. The profile of the tree was visualized with Tree View PPC version 1.6.5 (Institute of Biomedical and Life Sciences, Scotland, UK).

Nucleotide Sequence Accession Numbers

The nucleotide sequences in this study were submitted to Genbank and are available under the following accession numbers:

gag-p24 sequences: AY539583–AY539638 (n = 56)
pol-IN sequences: AY539639–AY539658 (n = 20)
env-C2V3 sequences: AY543545–AY543584 (n = 40)
env-gp41 sequences: AY541010–AY541028 (n = 19)

RESULTS

The epidemiologic and clinical data on the 57 HIV-1-infected individuals and genetic subtypes of HIV-1 strains identified in *gag*-p24, *env*-C2V3, *env*-gp41, and *pol*-IN are shown in Table 1.

Phylogenetic Analysis in the *env*-C2V3 Sequences

Out of the 57 samples, 40 (70.2%) were found to be positive for HIV-1 PCR with the set of *env*-C2V3 primers. The remaining 17 samples (29.8%) were negative for PCR with the *env*-C2V3 primers used in the present study. The phylogenetic tree based on *env*-C2V3 sequences (Fig. 1A) showed that 34 (85.0%) were probable subtype A, 1 (2.5%) F2, 4 (10.0%) CRF01_AE, and 1 (2.5%) U (unclassified). Thus, the majority of the samples fell into subtype A, though the clustering with subtype A reference sequences was not completely significant. We further analyzed these 34 probable subtype A sequences without the rest of the sample sequences (Fig. 1E), showing that 14 clustered with CRF02_AG reference strains, 7 with CRF11_cpx, and 1 with CRF13_cpx, significantly (with 87.0, 76.3, and 100% bootstrap values, respectively).

Phylogenetic Analysis in the *gag*-p24 Sequences

All of the 57 samples but one (02CM319) were found to be positive for HIV-1 PCR with the set of *gag* primers. The phylogenetic tree based on *gag* sequences (Fig. 1B) showed that 45 (80.4%) were subtype A, 6 (10.7%) B, 1 (1.8%) F2, 1 (1.8%) CRF09_cpx, and 3 (5.4%) CRF13_cpx. Out of