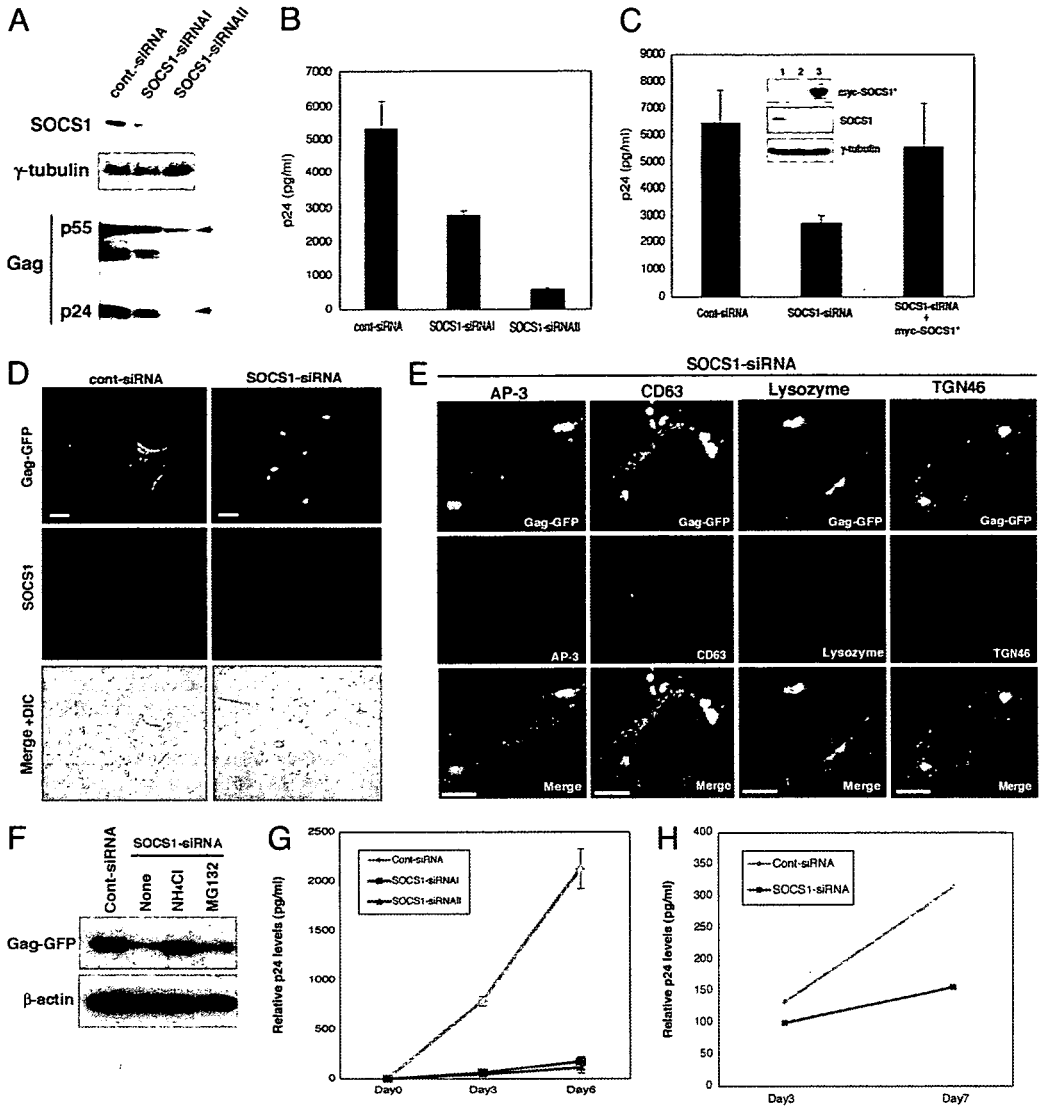

Fig. 4. The targeted inhibition of SOCS1 suppresses Gag trafficking and HIV-1 particle production and enhances Gag degradation in lysosomes. (*A* and *B*) 293T cells were transfected with either control siRNA or two different SOCS1-specific siRNAs (I or II) together with pNL4-3. At 48 h after transfection, cell lysates were subjected to immunoblotting analysis with the indicated antibodies (*A*). Cell supernatants were then subjected to ELISA analysis of p24 levels (*B*). (*C*) 293T cells were transfected with pNL4-3 and cotransfected with control-siRNA, SOCS1-siRNAI alone, or SOCS1-siRNAI plus siRNA-resistant myc-SOCS1 (myc-SOCS1*). After 48 h, cell supernatants were collected and subjected to p24 ELISA. (*Inset*) Immunoblots of the cell lysates. (*D*) HeLa cells were transfected with control or SOCS1-specific siRNA and cotransfected with GFP-Gag. At 48 h after transfection, the cells were subjected to confocal microscopy. (*E*) HeLa cells were transfected with Gag-GFP and SOCS1-siRNA constructs for 48 h. Cells were then fixed and subjected to immunofluorescent analysis with indicated antibodies followed by DAPI staining. (Scale bars: 10 mm.) (*F*) HeLa cells were transfected with Gag-GFP and cotransfected with either control-siRNA or SOCS1-siRNA. After 36 h, the cells were treated with a mock solution, 10 mM NH_4Cl or 10 μM MG132 for another 16 h. Cell were then harvested and subjected to immunoblotting analysis with anti-GFP or anti-β-actin antibodies. (*G*) Jurkat cells were infected with a retroviral vector encoding control (Cont) or two different SOCS1-specific siRNAs (I or II). After selection with puromycin, the cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points. (*H*) Human primary CD4 T cells were separated from healthy donors and infected with lentivirus vectors encoding either control- or SOCS1-siRNAI. The cells were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.1), and p24 antigen levels in cell supernatant were measured by ELISA at the indicated time points.

this time point, Gag-GFP was found to localize predominantly in a perinuclear region in the control cells (Fig. 3*C*), whereas almost half of the SOCS1-transfected cells exhibited Gag-GFP localization on PM (Fig. 3*D*). These results again indicate that SOCS1 efficiently enhances the trafficking of newly synthesized Gag protein to PM.

The Targeted Disruption of SOCS1 Inhibits Gag Trafficking and HIV-1 Particle Production. To delineate further the role of SOCS1 in the trafficking of Gag and in subsequent HIV-1 particle production, we depleted cellular SOCS1 by siRNA. The significant depletion of SOCS1 expression by two different SOCS1-specific siRNA constructs was confirmed by immunoblotting analysis (Fig. 4 *A* and *B*). Significantly, in cells cotransfected with pNL4-3 and SOCS1-specific siRNAs, both HIV-1 particle release and the levels of intracellular Gag protein are significantly decreased compared with the control cells (Fig. 4 *A* and *B*). Furthermore, the effects of SOCS1-siRNA on the inhibition of HIV-1 particle production was diminished by reexpression with a codon-optimized SOCS1 construct that is resistant to these siRNAs (Fig. 4*C*), indicating that the SOCS1 siRNA suppression of HIV-1 particle production depends on the availability of endogenous SOCS1.

Consistent with these observations, immunofluorescent analysis further revealed that the expression of SOCS1-siRNA dramatically inhibits Gag trafficking such that Gag proteins accumulate in the perinuclear regions as large solid aggregates, as has been reported (20) (Fig. 4*D*). This finding indicates that SOCS1 plays an essential role in the Gag trafficking from perinuclear clusters to PM. Interestingly, these discrete perinuclear clusters of Gag were found to colocalize with lysosome markers, lysozyme, and partly with AP-3, but neither with the late endosome MVB marker CD63 nor the *trans*-Golgi marker TGN46, indicating that Gag is targeted for degradation by lysosomes when the function of SOCS1 is inhibited (Fig. 4*E*). In support of this notion, the levels of intracellular Gag were found to be significantly increased by treatment with a lysosome inhibitor NH_4Cl but not by a proteasome inhibitor MG132 in SOCS1-siRNA cells (Fig. 4*F*), further indicating that the perinuclear clusters of Gag will undergo lysosomal degradation rather than proteasomal degradation when optimal Gag transport to PM is suppressed by the inhibition of SOCS1.

We next addressed whether targeted SOCS1 inhibition would affect HIV-1 particle production in human T cells. The effect of SOCS1 depletion was clearly evident in both HIV-1_{NL4-3}-infected

Jurkat cells and human primary $CD4^+$ T cells, which demonstrated pronounced decreases in virus particle production in SOCS1-siRNA-expressed cells compared with the controls (Fig. 4 *G* and *H*). These results together indicate that the specific inhibition of SOCS1 suppresses the optimal trafficking of Gag to PM, resulting in the degradation of Gag in lysosomes, which in turn leads to the efficient and reproducible inhibition of HIV-1 particle production in various types of human cells.

Discussion

In this work, we report that SOCS1 is an inducible host factor during HIV-1 infection and plays a key role in the late stages of the viral replication pathway via an IFN-independent mechanism (SI Fig. 6). These results represent evidence that SOCS1 is a potent host factor that facilitates HIV-1 particle production via posttranscriptional mechanisms.

SOCS1 has been shown to be a suppressor of several cytokine signaling pathways, and like all SOCS family members it has a central SH2 domain and a conserved C-terminal domain known as the SOCS box (21, 22). Structure–function analyses have further demonstrated that the SOCS1 SH2 domain is required for the efficient binding of its substrates (23, 24). Indeed, our current analyses have also revealed that the SH2 domain of SOCS1 is required for its interaction with the HIV-1 Gag protein. We have shown from our present data that the SOCS box is also required for SOCS1 to function during HIV-1 particle production.

The SOCS box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity (21, 25). Biochemical binding studies have shown that the SOCS box of SOCS1 interacts with the elongin BC complex, a component of the ubiquitin/proteasome pathway that forms an E3 ligase with Cul2 (or Cul5) and Rbx-1 (21, 26, 27). We show from our current experiments that the SOCS box is required for HIV-1 particle production, indicating the involvement of the ubiquitin/proteasome pathway. However, it is still unknown whether SOCS1 promotes the ubiquitination of Gag and, if so, whether the mono- or poly-ubiquitination of Gag would affect its trafficking and protein stability. Further studies will be necessary to clarify the biological significance of Gag ubiquitination.

Perlman and Resh (20) recently reported that newly synthesized Gag first appears to be diffusely distributed in the cytoplasm, accumulates in perinuclear clusters, passes transiently through a MVB-like compartment, and then traffics to PM. Consistent with these observations, our current work also shows that Gag is accumulated at perinuclear clusters as solid aggregates when its targeting to PM is impaired because of the SOCS1 inhibition.

Another aspect of SOCS1 function during HIV-1 infection was proposed recently. Song *et al.* (28) reported that SOCS1-silenced dendritic cells broadly induce the enhancement of HIV-1 Env-specific $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T helper cells as well as an antibody response. The induction of the SOCS1 gene in HIV-1 infected cells might therefore disrupt a specific intracellular immune response to HIV-1 in infected host cells.

Based on the strong evidence that we present in our current work that SOCS1 positively regulates the late stages of HIV replication, we conclude that SOCS1 is likely to be a valuable therapeutic target not only for future treatments of AIDS and related diseases, but also for a postexposure prophylaxis against disease in HIV-1-infected individuals.

Materials and Methods

Antibodies and Fluorescent Reagents. Antibodies and fluorescent reagents were obtained from the following sources. Anti-CD63, anti-AP-3, anti-myc (A-14), and anti-SOCS1 (H-93) were from Santa Cruz Biotechnology. Anti-SOCS1 was from Zymed Laboratories. Anti-FLAG (M2) and anti-HA (12CA5) were from Sigma and Roche Diagnostics, respectively. Anti-HIV-p24 (Dako; Cytomation), anti-STAT1, and anti-phospho-STAT1 (Y701) were from BD Transduction Laboratories. Sheep polyclonal anti-TGN46 was from GeneTex.

Plasmid Constructs. Expression constructs for SOCS1 have been described in ref. 29. GST fusion constructs with specific regions derived from the codon-optimized *gag* were generated (MA, CA, NC, p6, Δp6, full-length Gag) by cloning into pGEX-2T (GE Healthcare Bio-Sciences) as described in ref. 30. For retrovirus-mediated siRNA expression, pSUPER.retro.puro vector was digested, as described in ref. 31, with the following sequences: SOCS1-siRNAI, TCGAGCTGCTGGAGCACTA; SOCS1-siRNAII, GGCCAGAACCTTCCTCCTCTT; control siRNA, TCGTATGTTGTGTGGAATT.

Electron Microscopy. Transfected 293T cells were fixed with 2.5% glutaraldehyde and subjected to TEM, as described (14, 32).

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Anti-Retroviral Drug Resistance-Associated Mutations Among Non-subtype B HIV-1-Infected Kenyan Children With Treatment Failure

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Recently increased availability of anti-retroviral therapy (ART) has mitigated HIV-1/AIDS prognoses especially in resource poor settings. The emergence of ART resistance-associated mutations from non-suppressive ART has been implicated as a major cause of ART failure. Reverse transcriptase inhibitor (RTI)-resistance mutations among 12 non-subtype B HIV-1-infected children with treatment failure were evaluated by genotypically analyzing HIV-1 strains isolated from plasma obtained between 2001 and 2004. A region of *pol-RT* gene was amplified and at least five clones per sample were analyzed. Phylogenetic analysis revealed HIV-1 subtype A1 (n = 7), subtype C (n = 1), subtype D (n = 3), and CRF02_AG (n = 1). Before treatment, 4 of 12 (33.3%) children had primary RTI-resistance mutations, K103N (n = 3, ages 5–7 years) and Y181C (n = 1, age 1 year). In one child, K103N was found as a minor population (1/5 clones) before treatment and became major (7/7 clones) 8 months after RTI treatment. In 7 of 12 children, M184V appeared with one thymidine-analogue-associated mutation (TAM) as the first mutation, while the remaining 5 children had only TAMs appearing either individually (n = 2), or as TAMs 1 (M41L, L210W, and T215Y) and 2 (D67N, K70R, and K219Q/E/R) appearing together (n = 3). These results suggest that "vertically transmitted" primary RTI-resistance mutations, K103N and Y181C, can persist over the years even in the absence of drug pressure and impact RTI treatment negatively, and that appearing patterns of RTI-resistance mutations among non-subtype B HIV-1-infected children could possibly be different from those reported in subtype B-infected children. ***J. Med. Virol. 79:865–872, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: vertical transmission; anti-HIV resistance patterns; persistence of mutations; Kenya

INTRODUCTION

The emergence of anti-retroviral drug (ARV)-resistance mutations is a major cause of anti-retroviral treatment (ART) failure [D'Aquila et al., 1995; Lorenzi et al., 1999; Zolopa et al., 1999]. These drug-resistant HIV-1 strains can be transmitted through vertical, sexual, and parenteral routes [Erice et al., 1993; Conlon et al., 1994; Boden et al., 1999; Little et al., 1999; Brenner et al., 2000; Pillay et al., 2000; Salomon et al., 2000; Duwe et al., 2001]. Vertically transmitted multidrug resistant HIV-1 strain has been shown to persist for 9 months in an infant after postnatal therapy [Johnson et al., 2001]. Similarly, K103N-containing HIV-1 variants acquired after the administration of single dose-nevirapine, a non-nucleoside reverse-transcriptase inhibitor (NNRTI), have been reported to persist for more than 1 year in some women and infants after vertical transmission [Flys et al., 2005]. However, long-term persistence of vertically

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transmitted ARV-resistance mutations in the absence of drug pressure among infants and children is yet to be demonstrated.

Recently, the importance of ARV-resistant strains detected as minor populations has been reported. Minor drug-resistant HIV-1 populations have been detected both in the early phase of treatment failure [Coffin, 1995] and during successful structured treatment interruption [Metzner et al., 2003]. Minor drug-resistant populations undetectable by conventional assays can eventually overgrow and affect the clinical course [Dykes et al., 2004; Lecossier et al., 2005]. These minor drug-resistant populations have also been found to persist longer than expected previously in untreated patients, a favorable condition for wild-type virus to overgrow, which also indicates the risk of resistance transmission even from minor strains [Charpentier et al., 2004].

In patients experiencing treatment failure with nucleoside reverse-transcriptase inhibitors (NRTI), such as lamivudine plus either zidovudine or stavudine, the M184V mutation has been reported to always appear first, eventually followed by cumulative acquisition of thymidine-analogue-associated mutations (TAMs) if treatment with non-suppressive regimen is continued [Johnson et al., 2005]. Extensive studies on ARV-resistance suggest that HIV-1 may develop TAMs by either one of two distinct pathways; TAM 1 (M41L, L210W, and T215Y) or TAM 2 (D67N, K70R, and K219Q/E/N/R) [Flandre et al., 2003; Cozzi-Lepri et al., 2005]. However, most of these studies have focused on HIV-1 subtype B, which accounts for only 12% of the global HIV/AIDS pandemic, and data on non-subtype B HIV-1 is still limited. Furthermore, several differences in the development of ARV-resistance between subtype B and non-subtype B HIV-1 have been suggested [Apetrei et al., 1998; Quinones-Mateu et al., 1998; Pieniazek et al., 2000]. Most ARV-resistance studies have focused on adult populations [Yerly et al., 1998; de Ronde et al., 2001; Dykes et al., 2001; Brenner et al., 2002; Wainberg, 2003]. However, these findings may not be applicable directly to children, since several factors influencing selection of ARV-resistance such as pharmacokinetic properties; drug safety, tolerance, and antiviral activity of combination therapy, are usually different in the children [Kline et al., 1996].

The aim of this study was to investigate the patterns of emergence and the variable stability of ARV-resistance-associated mutations among non-subtype B HIV-1-vertically-infected children who developed eventually clinical failure with subsequent ART.

METHODS

Study Population

The subjects in this study resided in children's home in Nairobi, which housed 95 HIV-1-infected children. These children were born to HIV-1-infected mothers who either died of, or were too debilitated by HIV/AIDS hence could not offer basic care to the children. Of 95 children 55 were on ART as of August 2004. The duration of ART varied among children (mean: 23.3 months, range: 5–46 months). Of 55 children on ART 12 (8 males and 4 females, mean age: 7.4 years) experienced treatment failure, characterized by an initial decrease in plasma viral load (to undetectable level in one child) after treatment initiation and subsequent increase in the viral load as treatment continued. Seven of the 12 children received single ART regimen only during the study period: 5 received zidovudine/lamivudine/nevirapine, 1 zidovudine/didanosine/efavirenz, and 1 zidovudine/lamivudine/efavirenz (Table I). On the other hand, the remaining five children received multiple ART regimen during the study period: two received zidovudine/lamivudine/efavirenz followed by zidovudine/didanosine/efavirenz, two zidovudine/lamivudine/nevirapine followed by didanosine/lamivudine/efavirenz, and one didanosine/lamivudine/abacavir followed by zidovudine/didanosine/efavirenz and later didanosine/stavudine/efavirenz (Table I). These 12 children were admitted into the home by their first birthday and their HIV-1 status was confirmed serologically at 18 months of age. None of these children had history of previous exposure to any ARV.

This study was approved by the Kenya Medical Research Institute's National Ethical Review Committee on behalf of the Kenyan Government and conducted according to the national and international regulations governing the use of human subjects in biomedical research. The study was conducted within the continuing anti-retroviral, medical and healthcare programs of the institution without additional demand for blood samples solely for research purposes.

$CD4^+$ Cell Counts and Plasma Viral Loads

$CD4^+$ T cell counts of peripheral blood were determined using the FACSCOUNT (Becton-Dickinson, Beiersdorf, Germany) and plasma HIV-1 RNA loads using the Amplicor HIV-1 Monitor kit version 1.5 (Roche Diagnostics, Alameda, CA) with detection limit of 400 copies/ml according to the manufacturer's instructions.

Extraction and Amplification of Plasma HIV-1 Viral RNA

HIV-1 RNA was extracted from 100 µl of plasma using SMITEST EX-R and D (Sumitomo Metal Industries, Tokyo, Japan) according to the manufacturer's instructions. A region of the *pol-RT* gene (corresponding to nt 2480–3180 of HIV-1_{HXB2}) was amplified by both one-step RT-PCR (Invitrogen, Carlsbad, CA) and nested PCR with 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 [Ndembi et al., 2004; Songok et al.,

TABLE I. General Characteristics of Non-B Subtype HIV-1-Infected Study Children

Sample ID	Age* (years)/sex	HIV-1 subtype/CRF	Study point (month, year)	ART[a] (initiation time)	$CD4^+$ T cell count (/μl)	Plasma viral load (copies/ml)	NRTI[b]-resistance mutations	NNRTI[c]-resistance mutations
NYU30	11/F	A1	Jul '02	ZDV, 3TC, EFV (Jun '01)	456	<400		
			Mar '03		475	24,857		
			Jan '04	ZDV,DDI,EFV (May 03)	267	89,063	*D67N + K70R + K219Q*	*L100I*
NYU33	11/F	A1	Jul '02	ZDV, 3TC, EFV (Jun '01)	549	3,449		
			Mar '03	ZDV,DDI,EFV (Oct 01)	556	122,419	*K219Q*	*K101Q*
			Feb '04		690	6,457	*K219Q + D218E*	*K101Q*
NYU36	11/M	D	Oct '01	ddI,3TC,ABC (Apr '01)	309	114,754	*M184V + T215F*	
			May '02			880,405	*M184V + T215F*	*I178M*
			Aug '02	ZDV,DDI,EFV (Oct 01)	321	81,870	*M184V + T215F*	*G190A*
			Apr '03		279	607,224	*T215F*	*G190A*
			Feb '04	D4T,DDI,EFV (Nov 02)	458	393,420	*T215F*	*G190A*
NYU38	10/M	C	Mar '03	ZDV,3TC,NVP (Sep '02)	388	38,459	*D67N*	
			Dec '03		188	60,695	*D67N + K70R + L210W + K219E*	
			Feb '04	DDI,3TC,EFV (Mar 04)	157	38,211	*D67N + K70R + L210W + K219E*	
			Aug '04		149		*D67N + K70R + L210W + D218E + K219E*	
NYU44	9/M	A1	Feb '02		208	1,017,931		*K103N*
			Mar '03	ZDV, DDI, EFV (May '02)	370	71,895	*D67N + K70R + T215F + K219Q*	*K103N + G190A*
			Dec '03		474	150,549	*D67N + K70R + T215F + K219Q + M41L + V75M*	*K103N + G190A*
NYU62	8/M	A1	Dec '01		589	239,644		
			Sep '02	ZDV, 3TC, NVP (Sep '02)	828	2,838	*D67N + K70R*	*G190A*
			Mar '03		568		*D67N + K70R + T215F + K219E*	G190A
			May '04			6,901	*D67N + K70R + T215F + K219E*	*G190A + Y181C*
NYU69	6/M	A1	Mar '03		192	227,176		*K103N*
			May '04	ZDV, 3TC, NVP (Mar '03)	400	113,868	*M184V*	*K103N*
NYU70	7/M	D	Sep '02		718	700,563		*K103N*
			Jun '03	ZDV, 3TC, NVP (Jul '03)	169	1,323,431		*K103N*
			Dec '03		502	188,059	*K70R + M184V*	*K103N*
NYU79	6/M	A1	Feb '03	ZDV,3TC,NVP (Apr '03)	70	159,826		
			Feb '04		551	244,506	*V75M + M184V*	*K101E + G190A*
			Jun '04	DdI,3TC,EFV (Mar 04)	347	472,203	*V75M + M184V*	*K101E + G190A + Y181C*
NYU83	5/M	A1	May '01		876	634,644		
			Jul '02	ZDV, 3TC, EFV (May '04)	946	50,570	*M184V*	*K103N*
			Apr '03		1138	74,437	*M184V*	*K103N*
			Aug '04		1125	197,301	*M184V + T215Y*	*K103N*
NYU85	5/F	CRF02_AG	Feb '03		178	30,690		
			Dec '03	ZDV, 3TC, NVP (Apr '03)	1214	3,264	*D67N + M184V*	*K103N*
			Apr '04		1148	79,080	*D67N + M184V*	*K103N*
NYU90	2/F	D	Apr '03		6	523,950		*Y181C*
			Jan '04	ZDV, 3TC, NVP (Apr '03)	399	55,679	*M184V*	
			Mar '04		379	155,191		*K103N*

*As of August 2004.
[a]ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efivarenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.
[b]NRTI, nucleoside analogue RTI.
[c]NNRTI, non-nucleoside RTI; blank, no mutation detected.

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2004]. Amplification was done with 1 cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension of 72°C for 10 min. PCR amplification was confirmed by ethidium bromide staining of samples electrophoresed on an agarose gel.

Cloning, Sequencing, and Subtyping

The amplified products were cloned using the TOPO TA Cloning kit (Invitrogen) and sequenced as described previously [Ndembi et al., 2004; Songok et al., 2004]. The sample nucleotide sequences were aligned with HIV-1 subtype reference sequences from the Los Alamos database by CLUSTALW (version 1.81) with minor manual adjustments. Phylogenetic trees were constructed and visualized as described previously [Ndembi et al., 2004; Songok et al., 2004]. To improve the accuracy of HIV-1 subtyping, we used the genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/) as needed.

RTI Resistance-Associated Mutations

The RT nucleotide sequences (697 bps) were translated into the corresponding 232 amino acids and analyzed for previously reported drug resistance-associated mutations in subtype B strains using the Stanford university HIVdb sequence analysis program. For each sample, at least five clones were obtained and genotyped to detect the presence of minor populations.

RESULTS

General characteristics, treatment history, demographic, immunological, and virological data of the 12 HIV-1-infected children studied are summarized in Table I.

HIV-1 Subtypes

All children were infected with non-subtype B HIV-1: subtype A1 (n = 7), subtype C (n = 1), subtype D (n = 3), and circulating recombinant form (CRF)-02_AG (n = 1) (Table I).

RTI Resistance-Associated Mutations Before Treatment

Of the 12 children, 4 (33.3%) harbored NNRTI-resistance mutations before treatment. Three children, NYU44 (age, 7 years), NYU69 (5 years), and NYU70 (6 years), had K103N while NYU90 (1 year) had Y181C detected before treatment (Table I). All the mutations but one (one of seven clones in NYU69) were detected as full clones (Table IV). K103N detected in three children persisted, while Y181C detected in one child disappeared during treatment.

Emerging Pattern of NRTI Resistance-Associated Mutations

The patterns of NRTI-resistance mutations are summarized in Table II. M184V appeared as the first

TABLE II. Patterns of NRTI*-Resistance Mutations in Non-B Subtype HIV-1-Infected Children With Treatment

Child (ID)	Study point (mpti[a]) 1st	2nd	3rd	4th	5th	Treatment
NYU69	*M184V* (10)					ZDV/3TC
NYU90	*M184V* (9)					ZDV/3TC
NYU83	*M184V* (13)	*M184V* (22)	*M184V* + 1TAM[b] (38)			ZDV/3TC
NYU70	*M184V* + 1TAM (6)					ZDV/3TC
NYU85	*M184V* + 1 TAM (9)	*M184V* + 1TAM (12)				ZDV/3TC
NYU36	*M184V* + 1 TAM (6)	*M184V* + 1 TAM (13)	*M184V* + 1TAM (18)	1 TAM (24)	1 TAM (34)	DDI/3TC/ABC, ZDV/DDI, D4T/DDI
NYU62	2 TAMs (6)	4 TAMs (12)	4 TAMs (22)			ZDV/3TC
NYU44	4 TAMs (11)	5 TAMs + *V75M* (19)				ZDV/DDI
NYU33		1 TAM (23)	2 TAMs (34)			ZDV/3TC, ZDV/DDI
NYU30			3 TAMs (31)			ZDV/DDI
NYU38	1 TAM (8)	4 TAMs (15)	4 TAMs (17)	5 TAMs (23)		ZDV/3TC, DDI/3TC
NYU79	*M184V* + *V75M* (10)	*M184V* + *V75M* (13)				ZDV/3TC, DDI/3TC

*NRTI, nucleoside analogue RTI.
[a]mpti, months post treatment initiation.
[b]TAM, thymidine analogue-associated resistance mutation; blank, no mutation detected.

primary NRTI-resistance mutation in 3 of 12 children (NYU69, NYU90, and NYU83), (later followed by the acquisition of one TAM in NYU83), while M184V appeared as first primary NRTI-resistance mutation with one TAM in three children (NYU36, NYU70, and NYU85) who received zidovudine/lamivudine, zidovudine/didanosine, or lamivudine/didanosine. The remaining five children (NYU30, NYU33, NYU38, NYU44, and NYU62) had a mixture of TAMs appearing as first mutations. Three of them (NYU44, NYU62, and NYU38) had both TAM 1 (M41L, L210W, and T215Y) and TAM 2 (D67N, K70R, and K219Q) profiles detected together. M184V appeared as the first primary NRTI-resistance mutation together with V75M in child NYU79. NYU33 developed K219Q only, a "secondary" NRTI-resistance mutation.

Emerging Pattern of NNRTI Resistance-Associated Mutations

In four of the five children who received nevirapine (NYU69, NYU70, NYU85, NYU90) K103N appeared as the first primary NNRTI-resistance mutation, while in one (NYU62) G190A appeared as the first mutation (Table III). In two of the five children who received efavirenz (NYU44 and NYU 83) K103N appeared as the first NNRTI-resistance mutation, while in two children (NYU30 and NYU33) L100I and K101Q, respectively, appeared as the first NNRTI-resistance mutation. One child (NYU36) who received didanosine/lamivudine/abacavir with subsequent change to an efavirenz-containing regimen developed I178M as the first NNRTI-resistance mutation, which was replaced later by appearance of G190A.

One child (NYU79) developed K101E and G190A as first NNRTI-resistance mutations with nevirapine therapy and developed additionally Y181C when ART was changed to efavirenz-containing regimen during the study period.

In the remaining one child (NYU38) no known NNRTI-resistance mutation was detected despite receiving nevirapine—and later efavirenz-containing regimen (Table III).

Growth of Minor Mutant Virus Population into Major One

Five of 12 children had RTI-resistance mutations detected as minor virus populations, which subsequently grew into full clones (Table IV). In the remaining seven children no RTI-resistant mutation was detected as a minor population (data not shown).

RTI-resistance mutations, such as T215F in child NYU36, T215F in NYU44, D67N/K70R/T215F in NYU62, and K101Q/K219Q in NYU33, appeared as minor populations after initiation of treatment, which overgrew subsequently to major populations.

In one child (NYU69), K103N was found as a minor population (1/5 clones) before initiation of treatment and became major population (7/7 clones) 8 months after treatment.

TABLE III. Patterns of NNRTI*-Resistance Mutations Among Non-B Subtype HIV-1-Infected Children With Treatment

Child (ID)	Study point (mpti[a])						
	Pre-treatment	1st	2nd	3rd	4th	5th	Treatment
NYU69	*K103N* (−4)	*K103N* (10)					
NYU70	*K103N* (−10, −1)	*K103N* (11)					
NYU85		*K103N* (9)	*K103N* (12)				NEVIRAPINE
NYU62		*G190A* (6)	*G190A* (12)	*G190A* + *Y181C* (26)			
NYU90	*Y181C* (−0.25)		*K103N* (11)				
NYU38							
NYU83		*K103N* (12)	*K103N* (22)	*K103N* (38)			
NYU30				*L100I* (31)			
NYU44	*K103N* (−3)	*K103N* + *G190A* (10)	*K103N*+*G190A* (18)				
NYU33			*K101Q* (11)	*K101Q* (34)			EFAVIRENZ
NYU36			*I178M* (13)	*G190A* (16)	*G190A* (24)	*G190A* (34)	
NYU79		*K101E* + *G190A* (10)	*K101E* + *G190A* + *Y181C* (14)				
NYU38							

*NNRTI: non-nucleoside analogue RTI.
[a]mpti, months post treatment initiation; blank, no mutation detected.

TABLE IV. Evolution of Minor RTI-Resistance Mutant Populations Among Non-B HIV-1-Infected Children With Treatment

Child ID	Study point (months post treatment)	ART[a]	Plasma viral load (copies/ml)	NRTI[b]-resistance mutations	NNRTI[c]-resistance mutations
NYU36	1st (6)		114,754	**T215F (1/9)[d] + M184V (6/8)**	
	2nd (13)	DDI, 3TC, ABC	880,405	**T215F (1/8) + M184V (2/8)**	I178M (6/8)
	3rd (18)	ZDV, DDI, EFV	81,870	**T215F (9/9) + M184V (8/9)**	G190A (8/9)
	4th (24)		607,224	**T215F (5/5)**	G190A (5/5)
	5th (34)	D4T, DDI, EFV	393,420	**T215F (7/7)**	G190A (7/7)
NYU44	Pre-treatment		1,017,931		K103N (5/5)
	1st (10)	ZDV, DDI, EFV	71,895	D67N (5/5) + K70R (5/5) + **T215F (1/5) + K219Q (5/5)**	K103N (5/5) + G190A (5/5)
	2nd (17)		150,549	D67N (5/5) + K70R (5/5) + **T215F (5/5) + K219Q (5/5)** + **M41L (1/5)** + V75M (3/5)	K103N (5/5) + G190A (5/5)
NYU62	Pre-treatment		239,644		
	1st (6)	ZDV, 3TC, NVP	2,838	**D67N (1/5) + K70R (1/5)**	G190A(5/5)
	2nd (12)			**D67N (5/5) + K70R (5/5) + T215F (2/5)** + K219E (5/5)	G190A (5/5)
	3rd (26)		6,901	**D67N (5/5) + K70R (5/5) + T215F (2/5)** + K219E (5/5)	Y181C (4/5) + G190A (5/5)
NYU69	Pre-treatment		227,176		**K103N (1/5)**
	1st (10)	ZDV, 3TC, NVP	113,868	M184V (7/7)	**K103N (7/7)**
NYU33	1st (15)		3,449		
	2nd (23)	ZDV, 3TC, EFV	122,419	**K219Q (4/11)**	**K101Q (6/11)**
	3rd (34)	ZDV, DDI, EFV	6,457	**K219Q (14/14)** + D218E (14/14)	**K101Q (14/14)**

[a]ART, anti-retroviral therapy; ZDV, zidovudine; ddI, didanosine; EFV, efivarenz; NVP, nevirapine; 3TC, lamivudine; d4T, Stavudine.
[b]NRTI, nucleoside analogue RTI.
[c]NNRTI: non-nucleoside RTI, blank: no mutation detected.
[d]Number of clones with mutation/ total number of clones analysed; bold, minor RTI-resistant mutant populations that evolved.

DISCUSSION

In the current study, NNRTI resistance-associated primary mutations, K103N and Y181C, were found before ART in four (33.3%) of 12 HIV-1-vertically-infected Kenyan children with subsequent ART failure. Three children aged 5–7 years already had K103N mutation, while one child aged 1 year already had Y181C by the time ART was started. These children had no history of previous exposure to any ART or blood transfusion, suggesting that these drug-resistance mutations were transmitted vertically from their mothers. However, ART history of these children's mothers could not be confirmed, and the use of nevirapine to reduce transmission of HIV-1 from mother to child had not been started by the year 2002 in Kenya [NASCOP, 2002].

This is the first report on the long-term persistence of NNRTI-resistance mutation for upto 7 years in vertically HIV-1-infected children albeit in the absence of ART. The K103N mutation has been reported to have little impact on the replicative capacity of HIV-1, allowing K103N variants to persist as dominant species at the expense of the wild strains [Brenner et al., 2002]. Thus, these current findings emphasize the need for drug-resistance testing among HIV-1-infected children prior to starting any NNRTI-containing regimen to avoid earlier treatment failure.

The selection of some ARV-resistance mutations among minor HIV-1 populations after ART initiation has been reported previously [Coffin, 1995; Metzner et al., 2003; Charpentier et al., 2004; Dykes et al., 2004; Lecossier et al., 2005]. In this study, RTI-resistance mutations detected in five children as minor populations after ART initiation subsequently grew into major populations, resulting in ART failure. In addition, it is noted that a primary NNRTI-resistance mutation, K103N, was found in one of five HIV-1 clones from a drug-naïve Kenyan child (NYU69), and this minor drug-resistant virus became dominant (seven of seven clones) after 8-months ART, resulting in treatment failure. These findings indicate that minor ARV-resistant HIV-1 variants existing before therapy can also be an important cause of treatment failure, as suggested previously [Dykes et al., 2004; Lecossier et al., 2005; Johnson et al., 2006]. Standard genotyping methods can only detect more than 25% of the virus variants [Gunthard et al., 1998]. Therefore, in order to pick minor variant populations and pre-empt treatment failure, more sensitive detection methods for minor HIV-1 populations would be required [Edelstein et al., 1998; Gunthard et al., 1998; Grant et al., 2002; Schuurman et al., 2002; Malet et al., 2003; Shi et al., 2004; Palmer et al., 2005].

Results from this study suggest the possible existence of two different patterns of emergence or acquisition of the TAMs among children who receive thymidine-analogues such as zidovudine, lamivudine, and/or stavudine. Seven of the 12 children had an initial development of M184V mutation, followed by the cumulative acquisition of TAMs, consistent with previous studies of subtype

B HIV-1 [Johnson et al., 2005], which reported that TAMs always develop by either one of two distinct pathways, TAM1 (M41L, L210W, and T215Y) or TAM 2 (D67N, K70R, and K219Q/E/R), under the pressure of thymidine analogue-containing ARVs. The remaining five children, however, developed TAMs only without the initial appearance of M184V mutation. Additionally, three of these children developed both TAMs 1 and 2 members concurrently, discordant with previous reports [Flandre et al., 2003; Cozzi-Lepri et al., 2005]. One child (NYU33) developed K219Q and K101Q mutations only, after 2-year treatment with zidovudine, didanosine, and efavirenz. These two mutations have been previously grouped among the secondary RTI-resistance-associated mutations, unable to cause drug-resistance in the absence of other primary RTI-resistance-associated mutations such as K70R or T215F [Garcia-Lerma, 2005]. These findings therefore suggest the possible existence of different pathways for development of RTI-resistance in non-subtype B HIV-1-infected children, different from those reported in subtype B-infected individuals, and that secondary RTI-resistance-associated mutations namely K219Q and K101Q could independently cause ART resistance among non-subtype B HIV-1-infected children. Further studies are however needed in order to confirm these findings.

The K103N mutation has been reported as the most commonly selected NNRTI-resistance-associated mutation, usually appearing first [Johnson et al., 2005]. The results from the children who received nevirapine in this study agree with this observation. However, the children who received efavirenz developed a variety of NNRTI-resistance-associated mutations, such as L100I, K101Q, I178M, and G190A. This is the first report to show the possibility of the K101Q and I178M to appear as the first NNRTI-resistance mutations with efavirenz therapy. L100I, Y181C, and G190A have already been described [Johnson et al., 2005]. In addition, one child (NYU38) who received nevirapine and later efavirenz containing regimen did not have any NNRTI-resistance-associated mutation despite experiencing treatment failure, suggesting a possible difference in the initial selection of NNRTI-resistant mutations between non-subtype B and subtype B HIV-1-Infected children. However, considering recent reports on the association between a homozygous variant of multidrug-resistance transporter *C3435T* and good immune recovery [Saitoh et al., 2005], and the correlation of homozygous *CYP2B6* *6 with plasma efavirenz concentrations in HIV-1-infected individuals treated with efavirenz-containing regimen [Tsuchiya et al., 2004], further pharmacogenetic studies would also be needed to elucidate these phenomenon.

In conclusion, this study suggests a possible long-term persistence of "vertically transmitted" NNRTI-resistance mutations in the absence of drug pressure, that minor populations of RTI-resistant HIV-1 mutants may impact negatively on the outcome of ART, and that there is a possible difference in the pattern of appearance and profile of RTI-resistance mutations between non-subtype B and subtype B HIV-1-infected children. Further studies with large population size are needed to confirm these findings.

SEQUENCE DATA

GenBank accession numbers of the sequences reported in this study are DQ679541 to DQ679753 for *Pol-RT*.

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RANTES −28G Delays and *DC-SIGN* −139C Enhances AIDS Progression in HIV Type 1-Infected Japanese Hemophiliacs

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ABSTRACT

The relationships between host immune factors and HIV-1 disease progression are still in dispute. Unlike CCR5Δ32, which has been found to delay disease progression of HIV-1, there still remain several factors whose effect on the clinical course is unconfirmed. To clarify the relationships, we selected seven single-nucleotide polymorphisms (SNPs) out of the previously reported factors, namely, *RANTES* promoter −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* promoter −589T, and *DC-SIGN* promoter −139C/−336C, and examined these in Japanese HIV-1-infected hemophiliacs (n = 102). The genotypes were examined by the direct sequencing method, and the distributions of genotype and allelic frequencies were compared between two groups, slow progressors (n = 54) who did not develop AIDS more than 10 years after intravenous infection and others (progressors) (n = 48). The allelic frequency of *RANTES* −28G was significantly higher in slow progressors (0.185) than in the progressor group (0.074) [p = 0.023, OR = 0.35, 95% CI (0.142, 0.880)]. *DC-SIGN* promoter −139C appeared in progressors with significantly higher allelic frequency (0.333) than slow progressors [0.204, p = 0.040, OR = 1.95, 95% CI (1.039, 3.677)]. With *RANTES* −403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* −589T, and *DC-SIGN* −336C, no significant difference was observed in allelic frequencies between the two groups. These results suggest that *RANTES* −28G was associated with delayed AIDS progression, while *DC-SIGN* −139C was associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs.

INTRODUCTION

THE INFLUENCE OF HOST IMMUNE FACTOR POLYMORPHISMS on AIDS progression has continuously been discussed. Previous studies, including multicenter meta-analyses, have almost concluded that CCR5Δ32[1–4] is related to delayed AIDS progression. These studies have mainly focused on sexually transmitted populations and intravenous drug users (IDUs) among whites, though some cohorts included hemophiliacs.

For years, factors other than CCR5, such as CCR2, SDF1, RANTES, and interleukin-4 (IL-4), have been studied. However, none of them has led to a definitive conclusion as to whether they delay AIDS progression in HIV-1-infected individuals or not. It is of note that most of the recent reports[4–8] have analyzed sexually transmitted individuals or IDUs, and, more importantly, most of the studied populations were whites.

In contrast to those subjects, HIV-1-infected hemophiliacs in Japan form a rather homogeneous population. Historically, Japanese hemophiliacs were thought to be infected with HIV-1 between 1982 and 1985 through contaminated blood coagulant, which means the time and mode of infection were virtually identical. We previously reported the relationships between CCR5 promoter polymorphism and the clinical courses in HIV-1-infected Japanese hemophiliacs.[9] In the current study, we tried to clarify further the impact of host immune factor single nucleotide polymorphisms (SNPs) on HIV-1 disease progression in the same population. The SNP sites analyzed were *RANTES* (*CCL5*) promoter −28/−403, *RANTES* intron 1.1

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(*RANTES* In1.1), SDF1 (*CXCL12*) 3′ untranslated region (UTR) position −801, *IL-4* promoter −589, and *DC-SIGN* (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin, also known as *CD209*) promoter −139/−336.

RANTES, the most potent ligand for CCR5, can compete the entry of and thus suppress replication of HIV-1 R5 (macrophage–tropic) strains, which use CCR5 as coreceptor.[10,11] The polymorphism in the promoter region, −28G, was reported to be associated with slower AIDS progression in Japanese HIV-1-infected cases,[12] though it was not supported by studies in other races.[13,14] *RANTES* −403A was initially reported to retard AIDS progression in HIV-1-infected European-Americans.[8] However, it was also reported to increase the rate of HIV-1 disease progression in cooperation with *RANTES* In1.1C, which is in strong linkage disequilibrium with *RANTES* −403A.[13]

SDF-1, the only natural ligand for CXCR4, can prevent T-lymphocyte infection with X4 (T cell tropic) strains of HIV-1, which use CXCR4 as coreceptor, through its direct blockade effect[15,16] and following CXCR4 downregulation.[17–19] The influence of *SDF1* 3′-UTR position −801A (*SDF1* 3′A) on AIDS progression is still in dispute.

IL-4 −589T was once reported to lower viral load and slow the rate of AIDS progression in whites,[7,20] however, this was not supported by other studies.[21,22] Further analyses are needed to confirm this issue.

The SNPs in the *DC-SIGN* promoter region have been reported to affect infectivity of HIV-1,[23] and recently that of *Mycobacterium tuberculosis*[24] and the severity of Dengue disease[25] as well. As for HIV-1 infection, a relationship between *DC-SIGN* −336C and acceleration of the primary parenteral infection has been reported.[23] Since dendritic cells play an important role not only at the initial phase of mucosal infection but in later expansion and reservoir function, we also evaluated whether *DC-SIGN* promoter −139C and −336C, two of the recently identified SNPs, could affect AIDS progression.

MATERIALS AND METHODS

Subjects

Cryopreserved peripheral blood mononuclear cells (PBMCs) collected from 104 HIV-1-positive Japanese hemophiliacs were used. These patients were presumed to be infected with HIV-1 virtually at the same period, between 1982 and 1985, through contaminated unheated blood products, and were enrolled in the study and followed up until 1996 by the Research Committee on Prevention of Developing Illness and Therapy for HIV-1-infected Patients in Japan.[9] The samples analyzed in this study did not overlap those in the previous report.[12]

All the patients were evaluated for their clinical stages according to 1987 CDC criteria.[26] They were divided into two groups: one consisted of 55 patients who did not proceed to AIDS without any treatment until the year 1994 (designated as "slow progressors" and the other included 49 patients who developed AIDS by 1994 ("progressors"). In this context, "slow progressors" were defined as those who did not progress to AIDS 10 years after HIV-1 infection, and "progressors" were defined as those who progressed AIDS within 10 years. None of the patients had been treated with antiretroviral drugs or other drugs, such as interferon-α, glycyrrhizin, or organic germanium compound.

Polymerase chain reaction and sequencing

In the current study, we examined seven SNPs, such as *RANTES* promoter −28G/−403A, *RANTES* In1.1C, *SDF-1* 3′A, *IL-4* promoter −589T, and *DC-SIGN* promoter −139C/−336C. To genotype the SNPs, we extracted genomic DNA from the cryopreserved PBMC using a DNA extraction kit (Qiagen, Hilden, Germany), amplified the target DNA by polymerase chain reaction (PCR), and did direct sequencing.

For the analysis of *RANTES* promoters, we amplified the target DNA by PCR with primers RA1 (5′-AGAAGGCCTTACAGTGAGA-3′) and RA3 (5′-GCGCAGAGGGCAGTAGCAA-3′).[12] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 49.2°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *RANTES* In1.1, we used 5′-CCTGGTCTTGACCACCACA-3′ and 5′-GCTGACAGGCATGAGTCAGA as forward and reverse primers, respectively.[13] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *SDF1*, 5′-CAGTCAACCTGGGCAAAGCC-3′ was used as the forward primer and 5′-AGCTTTGGTCCTGAGAGTCC-3′ as the reverse primer.[5] Amplification was done with one cycle of 95°C for 10 min and 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For the *IL-4* promoter 4-1 (5′-GAATTCAATAAAAAACAA-3′) was used as the forward primer and 4-1190 (5′-GAAACAGAGGGGAAGCA-3′) as the reverse primer.[27] Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 49.2°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. For *DC-SIGN* promoters, we designed a new primer set, PromF 5′-ACCTGACTACCCTAGGCATT-3′ (nt position −499 to −480) and PromR 5′-GGCCACAGCTTTTATTTCCC-3′ (nt position −38 to −57), and used them as forward and reverse primers, respectively. Amplification was done with one cycle of 94°C for 10 min and 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min.

PCR was performed with an AmpliTaq Gold PCR kit (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer instruction. All the amplified products were purified with Montage PCR (Millipore Co., Bedford, MA) and then sequenced by the dye terminator method using BigDye v1.1 and ABI 310 (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's instructions. All the sequence reactions except *RANTES* and *IL-4* were done with diluted PCR primers. For the sequencing of *RANTES* −403, RA2F (5′-ACTGATGAGCTCACTCTAGATG-3′)[12] was used as a primer. For the sequencing of the *IL-4* promoter, we designed another set of primers, 5′-GCCAAGGGCTTCCTTATGGGTAA-3′ (nt position −700 to −678) as forward primer and 5′-AATGCAGTCCTCCTGGGGAAAG-3′ (nt position −402 to −423) as reverse one.

Sample analysis

Genotypic distribution and allelic frequency of the SNPs were compared between the two groups, slow progressors and

progressors, with the c2 test or Fischer's exact test. A p value less than 0.05 was considered to be statistically significant. To confirm the associations between the SNPs and disease progression, odds ratios and 95% confidence intervals were further calculated by using unconditional logistic regression (SPSS 14.0J Regression Models).

RANTES haploypes were analyzed by an Expectation-Maximization algorithm utilizing Arlequin ver.3.01 (Genetica and Biometry Laboratory, Geneva, Switzerland).

RESULTS

The genotypic distribution and allelic frequency were analyzed for the SNPs, such as *RANTES* promoters −28G/−403A, *RANTES* In1.1C, *SDF-1* 3'A, *IL-4* −589T, and *DC-SIGN* promoters −139C/−336C, and compared between slow progressors and progressors in Japanese HIV-1-infected hemophiliacs. These results are shown in Tables 1 and 2. As for the SNPs in the *RANTES* promoter and intron, haplotype analysis (−403/−28/In1.1) was also done (Table 3). All the genotypes were in Hardy–Weinberg equilibrium.

Genotype distribution analysis

In the genotypic analysis of *RANTES* −28, there was a weak tendency that C/G and G/G genotypes were more frequent in slow progressors (p = 0.08). Besides, the G/G genotype was detected only in slow progressors, though the number was small (three cases).

DC-SIGN −139 T/C and C/C genotypes appeared more frequently in progressors, though the difference was not significant (p = 0.10). As for *DC-SIGN* −336, the C/C genotype was not detected in the current study.

RANTES −403, *RANTES* In1.1, *SDF-1* 3'A −801, and *IL-4* −589 showed no significant difference in genotype distribution between the two groups.

Allelic frequency analysis

RANTES promoters. The allelic frequency of *RANTES* −28G was 0.185 in slow progressors. It was significantly higher compared with that of progressors (0.074) [p = 0.023, OR = 0.35, 95% CI (0.142, 0.880)]. The allelic frequency of *RANTES* −403A was also higher in slow progressors (0.343) than in progressors (0.271). There was, however, no statistically significant difference [p = 0.29, OR = 0.71, 95% CI (0.391, 1.230)].

RANTES In1.1. The allelic frequency of *RANTES* In1.1C was 0.330 in slow progressors. It was higher than that of progressors (0.245), though this difference was not significant [p = 0.21, OR = 0.66, 95% CI (0.353, 1.222)].

RANTES haplotype. Four haplotypes, I (ACC), II (ACT), III (AGC), and IV (GCT), were detected in Japanese hemophiliacs (Table 3), and their frequencies were compared between the progressor and slow progressor groups. Haplotype III (A/G/C at RANTES −403/−28/In1.1, respectively) was higher in slow progressors (0.186) than in progressors (0.065), though the difference was not statistically significant (p = 0.052).

SDF1 3'-UTR. As for the allelic frequency of *SDF-1* 3'A, there was no significant difference between slow progressors

TABLE 1. ASSOCIATION BETWEEN GENOTYPES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Polymorphism		*Genotype distribution (cases)*		p *value*
		Slow progressors	*Progressors*	*(Fisher's exact test)*
RANTES -28	C/C	37	40	
(n = 101)	C/G	14	7	0.08
	G/G	3	0	
RANTES -403	G/G	24	25	
(n = 101)	G/A	23	20	0.48
	A/A	7	3	
RANTES In1.1	T/T	24	25	
(n = 100)	T/C	23	21	0.21
	C/C	6	1	
SDF1 -801	G/G	24	21	
(n = 102)	G/A	24	23	0.96
	A/A	6	4	
IL-4 -589	C/C	5	5	
(n = 100)	C/T	23	22	0.84
	T/T	25	20	
DC-SIGN -139	C/C	3	5	
(n = 102)	C/T	16	22	0.10
	T/T	35	21	
DC-SIGN -336	C/C	0	0	
(n = 101)	C/T	4	4	1.00
	T/T	50	43	

TABLE 2. ASSOCIATION BETWEEN ALLELIC FREQUENCIES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Polymorphism		*Allelic frequency*		p *value*	*Odds ratio (95% CI*
		Slow progressors	*Progressors*		
RANTES -28 (n = 101)	C	0.815	0.926	0.023	0.35 (0.142, 0.88)
	G	0.185	0.074		
RANTES -403 (n = 102)	G	0.657	0.729	0.29	0.71 (0.391, 1.230)
	A	0.343	0.271		
RANTES In1.1 (n = 100)	T	0.670	0.755	0.21	0.66 (0.353, 1.222)
	C	0.330	0.245		
SDF1 -801 (n = 102)	G	0.667	0.677	0.88	0.95 (0.531, 1.713)
	A	0.333	0.323		
IL-4 -589 (n = 100)	C	0.311	0.340	0.76	0.88 (0.484, 1.584)
	T	0.689	0.660		
DC-SIGN -139 (n = 102)	C	0.204	0.333	0.40	1.95 (1.039, 3.677)
	T	0.796	0.667		
DC-SIGN -336 (n = 101)	C	0.037	0.043	1.00[a]	1.16 (0.281, 4.754)
	T	0.963	0.957		

[a]Fisher's exact test.

(0.333) and progressors (0.323) [p = 0.88, OR = 0.95, 95% CI (0.531, 1.713)].

IL-4 promoter. The allelic frequency of *IL-4* −589T was 0.689 in slow progressors and 0.660 in progressors. There was no significant difference between the two groups [p = 0.76, OR = 0.88, 95% CI (0.484, 1.584)].

DC-SIGN promoters. The allelic frequency of the *DC-SIGN* promoter −139C was 0.333 in progressors. It was significantly higher than that of slow progressors [0.204, p = 0.040, OR = 1.95, 95% CI (1.039, 3.677)]. The allelic frequency of the *DC-SIGN* promoter −336C was 0.037 in slow progressors and 0.043 in progressors, yielding no significant difference [p = 1.00, OR = 1.16, 95% CI (0.281, 4.754)].

To validate these univariate associations, we further analyzed all the SNPs chosen in a multivariate manner using an SPSS regression model. The same SNPs were found to be significantly and nonsignificantly associated with clinical outcomes of HIV-1-infected Japanese hemophiliacs (Tables 2 and 4).

DISCUSSION

In the current study, *RANTES* −28G was found to be associated with delayed disease progression in HIV-1-infected Japanese hemophiliacs. This result supports the previous report, in which the −28G mutation increased RANTES expression and secretion, and thus was concluded to retard AIDS progression in Japanese HIV-1-infected individuals including hemo-

TABLE 3. RANTES HAPLOTYPE AND THEIR ASSOCIATIONS WITH CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS

Haplotype -403/-28/In1.1	*Slow progressors*		*Progressors*		*Overall*	
	No. of alleles	*Frequency (%)*	*No. of alleles*	*Frequency (%)*	*No. of alleles*	*Frequency (%)*
I ACC	13	(12.7)	16	(17.4)	29	(15.0)
II ACT	1	(1.0)	2	(2.2)	3	(1.5)
III AGC	19	(18.6)	6	(6.5)	25	(12.9)
IV GCT	69	(67.7)	68	(73.9)	137	(70.6)
	102		92		194	

TABLE 4. ASSOCIATION BETWEEN ALLELES OF HOST IMMUNE FACTORS AND CLINICAL OUTCOMES OF HIV-1-INFECTED JAPANESE HEMOPHILIACS (N = 102)

Allele	*Progressor (cases)*	*Slow progressor (cases)*	*Odds ratio*	*95% CIs*[a]
RANTES -28				
Non-G	41	37	1.000	
G	7	17	0.185	(0.051, 0.635)
RANTES -403				
Non-G	3	7	1.000	
G	45	47	2.371	(0.485, 11.595)
RANTES In1.1				
Non-C	26	25	1.000	
C	22	29	1.489	(0.544, 4.079)
SDF1 -801				
Non-G	4	6	1.000	
G	44	48	2.049	(0.484, 8.682)
IL-4 -589				
Non-C	21	26	1.000	
C	27	28	1.109	(0.468, 2.630)
DC-SIGN -139				
Non-C	21	25	1.000	
C	27	19	3.793	(1.451, 9.916)
DC-SIGN -336				
Non-C	44	50	1.000	
C	4	4	0.488	(0.093, 2.560)

[a]CIs, confidence intervals.

philiacs.[12] While its delaying effect was evaluated only by the decreased CD4 depletion rate in the previous study, a direct relationship between the allelic frequency and clinical outcomes could be observed in the present study. Moreover, *RANTES* −28 G/G homozygotes were found only in the slow progressor group.

RANTES In1.1C was reported to contribute to the rapid progression of AIDS in European-Americans and particularly in African-Americans.[13] In our study, however, the SNP was not found to influence disease progression in Japanese hemophiliacs. Our haplotype analysis showed that *RANTES* −28G, which was found to have AIDS-delaying effect, was always accompanied by *RANTES* In1.1C. The frequency of haplotype III, which contains both mutant alleles −28G and In1.1C, was significantly higher in Japanese hemophiliacs (0.129) than those reported in European-Americans (0.025) and in African-Americans (0.002).[13] In Japanese HIV-1-infected hemophiliacs, therefore, the protective effect of *RANTES* −28G might exceed the detrimental effect of *RANTES* In1.1C.

SDF-1 3′A was first reported to be associated with delayed onset of AIDS,[28] which was followed by conflicting reports,[6,29–31] concluding that *SDF-1* 3′A does not retard HIV-1 disease progression, either early or late in the course of infection. Our result was concordant with those of the reports denying the association, though Modi *et al.*[32] recently reported its protective effect as haplotype.

The polymorphism, *IL-4* promoter region −589T, was reported to be associated with delayed disease progression in HIV-1-infected nonhemophiliac whites.[7] In contrast, *IL-4* −589T was also reported to be associated with X4 strain acquisition,[27] which could lead to AIDS progression. In the current study, no significant difference was observed in allelic frequency of *IL-4* −589T between slow progressors and progressors. These results may be due to the bilateral functions of IL-4 in HIV-1 infection; it may suppress the primary infection of HIV-1 by downregulation of CCR5[33] and promote coreceptor switch by upregulation of CXCR4 as well.[34]

DC-SIGN is known to bind to HIV-1 gp120 and enhance *in trans* infection of HIV-1 from dendritic cells to T cells.[35] Recently, it was reported that an SNP in the promoter region of *DC-SIGN*, −336C, was associated with increased susceptibility to HIV-1 parenteral infection and not to mucosal infection among European-Americans.[23] In our study, however, the influence of *DC-SIGN* −336C on disease progression to AIDS was not observed. The allelic frequency of −336C was too low for statistical evaluation in Japanese hemophiliacs. Unexpectedly, *DC-SIGN* −139C was found to be associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs. These results may be explained by the report[36] that the SNP is located in the vicinity of a candidate binding site of transcription factor AP-1 (activator protein-1) in the *DC-SIGN* promoter region. The nucleotide substitution near the transcription factor-binding site in the promoter region may increase *DC-SIGN* expression level, resulting in the acceleration of AIDS progression.

In conclusion, our results suggest that *RANTES* promoter −28G is associated with delayed AIDS progression and *DC-SIGN* promoter −139C with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs, while *SDF-1* 3′-UTR, *RANTES* −403A, *IL-4* −589T, and *DC-SIGN* −336C do not

influence clinical courses. Further analysis is needed, particularly concerning the relationship among *DC-SIGN* promoter SNPs, modified *DC-SIGN* expression level, and the clinical course of HIV-1 disease.

SEQUENCE DATA

GenBank accession numbers of the sequences reported in this study are as follows: *RANTES* −28 (rs2280788), *RANTES* −403 (rs2107538), *RANTES* In1.1 (rs2280789), *SDF-1* −801 (rs1801157), *IL-4* −589 (rs2243250), *DC-SIGN* −139 (rs2287886), and *DC-SIGN* −336 (rs4804803).

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Molecular Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) and HIV-2 in Yaoundé, Cameroon: Evidence of Major Drug Resistance Mutations in Newly Diagnosed Patients Infected with Subtypes Other than Subtype B[▽]

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Prior to current studies on the emergence of drug resistance with the introduction of antiretroviral therapy (ART) in Cameroon, we performed genotypic analysis on samples from drug-naïve, human immunodeficiency virus (HIV)-infected individuals in this country. Of the 79 HIV type 1 (HIV-1) *pol* sequences analyzed from Cameroonian samples, 3 (3.8%) were identified as HIV-1 group O, 1 (1.2%) was identified as an HIV-2 intergroup B/A recombinant, and the remaining 75 (95.0%) were identified as HIV-1 group M. Group M isolates were further classified as subtypes A1 ($n = 4$), D ($n = 4$), F2 ($n = 6$), G ($n = 12$), H ($n = 2$), and K ($n = 1$) and as circulating recombinant forms CRF02_AG ($n = 41$), CRF11_cpx ($n = 1$), and CRF13_cpx ($n = 2$). Two *pol* sequences were identified as unique recombinant forms of CRF02_AG/F2 ($n = 2$). M46L ($n = 2$), a major resistance mutation associated with resistance to protease inhibitors, was observed in 2/75 (2.6%) group M samples. Single mutations associated with resistance to nucleoside reverse transcriptase inhibitors (T215Y/F [$n = 3$]) and nonnucleoside reverse transcriptase inhibitors (V108I [$n = 1$], L100I [$n = 1$], and Y181C [$n = 2$]) were observed in 7 of 75 (9.3%) group M samples. None of the patients had any history of ART exposure. Population surveillance of transmitted HIV drug resistance is required and should be included to aid in the development of appropriate guidelines.

The current standard for antiretroviral drug therapy (ART) in developed countries is the combination of two nucleoside reverse transcriptase (RT) inhibitors (NRTIs) plus a nonnucleoside RT inhibitor (NNRTI) or a protease inhibitor (PI). Since the successful trials in the late 1990s, combination ART has benefited and continues to aid many human immunodeficiency virus type 1 (HIV-1)-infected patients in developed countries, and it is becoming increasingly available in resource-constrained countries (17, 20, 24, 29, 30).

In countries with multiple antiretrovirals (ARVs) readily available, the prevalence of drug-resistant variants has ranged from 10 to 20% among drug-naïve patients (33), while in resource-constrained areas, resistance in the untreated HIV-infected population is rarely reported (23, 31). Recent interventions through such programs as the World Health Organization (WHO)'s 3 by 5 plan to treat 3 million people by the end of 2005 (33a) and the President's Emergency Plan for AIDS Relief have promoted significant access to ART in low- and middle-income countries. As of June 2005, about 500,000 people in sub-Saharan Africa were receiving ART, although the regional coverage rate was still 11% of the estimated number of patients with CD4 cell counts of ≤300/ml (2% of all HIV-infected patients in this region) (33a). Developing countries, including Cameroon, are moving towards universal access to HIV prevention, care, and treatment for those in need and at high risk of infection. This has led to the widespread use of antiretroviral drugs through structured national ART scale-up plans. Because of the complexity and open-ended duration of HIV treatments and the need to begin programs to treat many patients quickly, fears have been raised that emergence of ARV resistance may become a serious public health concern and render anti-HIV drugs useless. To assist ART programs and to minimize the emergence and transmission of HIV drug resistance strains and their public health consequences, WHO has developed a minimum-resource strategy for the surveillance and monitoring of HIV drug resistance in resource-limited countries. In Kenya, for example, where ART has been provided for 12 to 17% of the estimated need, the prevalence of resistant strains among drug-naïve patients has recently risen from 1% (2002) to more than 5% (2003) (WHO, personal communication). In Botswana, where treatment is available to all patients with <300 CD4 cells/ml, the prevalence of major mutations conferring PI resistance was estimated to be 4% among drug-naïve patients (4).

Unlike the case in southern and eastern African countries, where one or two HIV-1 subtypes dominate (22), all major groups and subtypes of HIV-1 cocirculate in Cameroon (1, 6, 14–19, 21, 22, 24, 28, 34–38). According to WHO/UNAIDS, as of the end of 2004, the prevalence of HIV-1 infection was estimated to be 4.8% overall and 9.8% for adults. To date,

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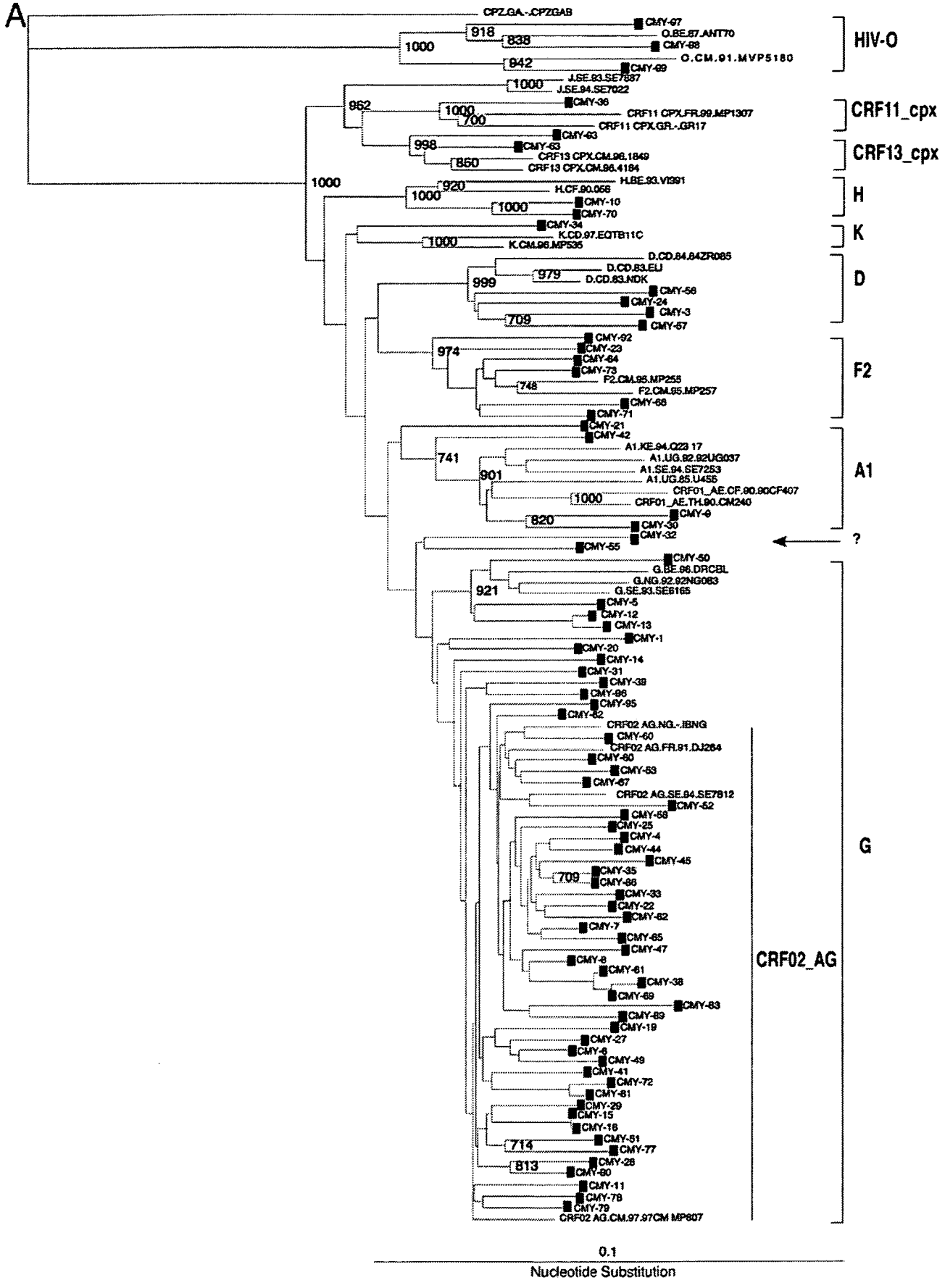

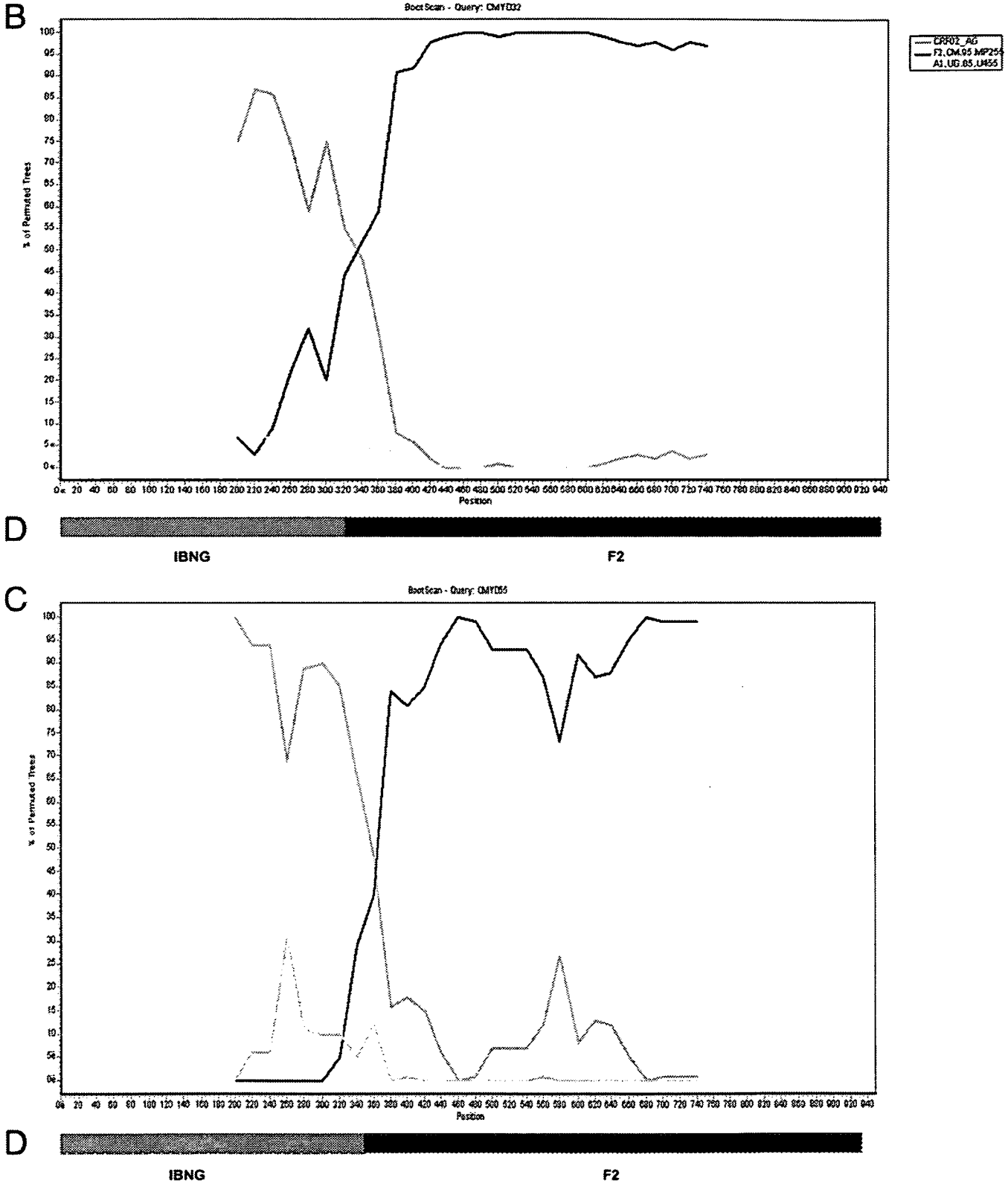

there have been several reports on the prevalence of ARV resistance mutations in the drug-naïve HIV-1-infected population of Cameroon (1, 6, 14–16, 19, 31). Baseline information on the frequency and types of ARV resistance mutations in Cameroon will help to inform optimal ART and enable the government to monitor the success of the national AIDS treatment program.

ART in Cameroon is based on the WHO guidelines, i.e., the