MOLECULAR AND PHENOTYPIC CHARACTERISTICS OF NEUROTROPIC HIV-1 SUBTYPE E

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Abstract. Although HIV-1 subtype E associated with neurological dysfunction is common, the virological characteristics of HIV-1 isolated from the CNS for this subtype have not yet been identified. In this study, paired blood and CSF isolated from patients with AIDs-defining illnesses were cultured, sequenced and aligned. Phylogenetic tree and nucleotide-distances from both blood and CSF were investigated. Cytopathicity and co-receptor usage of paired blood and CSF isolates were compared to define the specific characteristics of CNS isolates. The results confirmed that CSF isolates showed less cytopathicity. It was found that both blood and CSF isolates used either CXCR4 or CXCR4 and CCR5 as co-receptors. Interestingly, one CSF isolate using CCR3 as a co-receptor was identified. By sequence analysis, the pair-wise distances of envelope gp 120 sequence and those of all variable regions (except V3 region) between blood and CSF isolates were significantly different. The genetic distances in V1/V2 regions of CSF isolates showed more diversity than those of blood isolates. These findings suggest that the evolution of V1/V2 regions of CSF isolates seems to be an advantage for HIV-1 in CNS infection. In contrast, the genetic distance in V4 and V5 regions of CSF isolates showed less diversity, suggesting that conservation in these regions might be necessary during the process of HIV-1 CNS infection.

INTRODUCTION

A very large percentage of AIDS patients suffer from associated neurological complications. Many of these complications can be attributed to HIV-1 infection *per se* rather than to opportunistic infection or malignancies (Miller and Meucci, 1999). It is clear that effective replication of the virus does occur in the central nervous system (CNS) and that the brain is considered to be a significant reservoir for HIV-1 following primary infection (Lipton and Gendelman, 1995); HIV-1 seems to enter the brain by association with infected macrophages soon after infection (Edinger *et al*, 1997). It is now clear that all of the major cell types in the brain (neurons, glia and microglia) possess chemokine receptors (Lavi *et al*, 1998). A large number of chemokine receptors, including CXCR4, CCR3, CCR5, CCR8, CCR9/10 and CX3CR1 has been shown to exist in the brain or on brain-derived cells. The pattern of chemokine receptor expression in the brain is likely to determine the tropism of HIV- 1 for particular target cells (Gabuzda *et al*, 1998). Once HIV-1 has been transmitted to the brain compartment, a neurotropic strain may exist. In the CNS, there is certain genetic difference between blood- and brain- derived isolates (Keys *et al*, 1993; Korber *et al*, 1994). It is possible that HIV-1 variants specifically adapt to cells in the CNS either by independent evolution within the brain compartment or by selective

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recruitment from circulating virions (Singh *et al*, 1999). So far no molecular biological data on CNS isolates of HIV-1 subtype E, which is the predominant subtype in Thailand and Southeast Asian countries (McCutchan *et al*, 1996), have been reported. Therefore, studies of the molecular genetics of both blood and CSF isolates of HIV-1 subtype E from infected patients with no antiretroviral treatment were carried out. In addition, the cytopathicity and co-receptor usage of HIV-1 subtype E were determined.

MATERIALS AND METHODS

Patients

Ninety HIV-1 subtype E infected patients with AIDS-defining illness admitted to Bamrasnaradura Hospital were enrolled in the study. Cryptococcal meningitis, tuberculosis (TB) and *Pneumocystic carinii* pneumonia (PCP) were the opportunistic infections most commonly diagnosed. All but one patient (no. 95) had not been treated with any antiretroviral drugs. The major route of HIV-1 transmission to these patients had been heterosexual contact.

Samples processing and culturing

Paired blood and CSF samples were collected from HIV-1 infected patients who had given thier informed consent. The peripheral blood mononuclear cells (PBMCs) from infected patients were separated and co-cultured with phytohemagglutinin (PHA)-stimulated PBMCs from seronegative donors (according to the WHO standard protocol). For CSF, the cells and cryptococcal organisms were eliminated by filtration through a 0.2 μm millipore membrane. The cell free supernatant was used for culturing as described above. HIV replication was monitored by using a p24 antigen test kit (Organon Teknika, WI).

Cells and culture condition

MT-2 and SK-N-MC (neuroblastoma cell line) obtained from ATCC (American Type Culture Collection), U87/CD4/CXCR4, U87/CD4/CCR5 and Ghost CCR3 cell lines obtained from WHO (The Centralised Facility for AIDS Reagent) were rapidly thawed and maintained in 10% DMEM medium. All cell lines were seeded in a 24-well plate (Nunc A/S, Roskilde) of $5x10^5$ cells/well for 24 hours before challenge and overnight infection with 100 $TCID_{50}$ and then washed and maintained for two weeks. Viral replication was detected by p24 reagent test kit (Organon, Teknika, WI). The presence of multinucleated giant cells was observed by light microscope.

PCR amplification

Infected PBMCs were lysed by proteinase K and heat inactivated. Primary PCR was performed with the upstream primer 5'AGA AAG AGC AGA CAG TGG CAA and the downstream primer 5'GAA ATT CAA AGG TGA GTA TCC CTG. The mixture was subjected to 35 cycles of: 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes. For nested PCR, the upstream primer 5'GGG ATC CTT ATT ATG GGG TTC ATG TGT and the downstream primer 5'GGA ATT CTT TCC AGG TCT GAA were used. The primary PCR products were further amplified by the same process, except that the annealing temperatures were 55°C for 1 minute. The nested PCR products of *env* gp120 gene were 1,115 bases.

DNA sequencing

The PCR products were purified (Qiagen, CA), and sequenced with Dye-Terminator Cycle Sequencing Ready kit (Applied Biosystems Inc, USA) according to the manufacturer's method. The sequencing reaction was performed in an automated thermal cycler (Perkin-Elmer 2400, USA). After DNA extraction, the nucleotide sequences were determined by using an automated DNA sequencer (ABI 310; PE Applied Biosystem, USA). Both DNA and amino acid sequences of blood and CSF isolates were aligned with the MegAlign program (DNAstar, lasergene99, USA). The phylogenetic tree was constructed to confirm the subtype of HIV-1. The statistical difference of the genetic dis-

tance between paired blood and CSF sequences was determined by using paired *t*- test (SPSS program version 7.5).

Nucleotide sequence accession numbers

The DNA sequences of the HIV-1 strains determined in this study have been deposited in GenBank under accession numbers AF 322195-322214.

RESULTS

HIV-1 culture

Co-culture of peripheral blood mononuclear cells (PBMCs) from the blood of the 90 infected patients was done, most samples showed p24 antigen-positive on day 7. However, after 21 days of infection, 88 blood-derived isolates could be achieved (97.7%). Using filtered CSF for HIV-1 culture, the yield of CSF positive culture was 12/90 (13.3 %). Eleven of twelve HIV-positive CSF samples were associated with cryptococcosis; the one other sample was associated with TB. Ten CSF isolates (BC12, BC16, BC27, BC29, BC40, BC52, BC54, BC68, BC89 and BC95) were identified as subtype E while the other two isolates were subtype B. These ten paired blood and CSF isolates were used for further molecular and biological studies.

Specific characteristics of CNS isolates

The CSF isolates showed less cytopathicity than blood isolates. Four of ten blood isolates (BB27, BB29, BB52 and BB95) induced syncytium formation while one of the CSF isolates (BC52) with syncytium inducement (SI) was observed. In these SI isolates, the positive net charges ranged from 2 to 5. The basic amino acid substitution at position 11 of V3 loop was found in BB27 and BB95 isolates. However the predictive motif of SI phenotype, GPGR, was found only in the BB95 isolate.

Neurotropic infectivity and its sequence

Among 12 paired blood and CSF samples, only HIV-1 from the blood of patient no. 52 (BB52) could infect the neuroblastoma cell line (SK-N-MC). However, the difference of sequence divergence in V3 region between HIV isolates obtained directly from blood and from the neuroblastoma cell line after infection was relatively great.

Co-receptor usage

The results showed that both blood and CSF isolates could use CXCR4 or CXCR5 or both co-receptors. For HIV-1 in blood, 50%, 10% and 40% of isolates were shown to use CXCR4, CCR5 or both as co-receptors respectively. Similar to the co-receptor usage of HIV-1 in CSF, 40%, 10% and 40% of isolates used CXCR4, CCR5 or both respectively. Interestingly, one of the CSF isolates (BC89) used neither CXCR4 nor CCR5 but used CCR3 as a co-receptor. Of the correspondence subjects, seven of ten paired blood and CSF isolates used co-receptors in a different pattern; the remaining isolates showed the same pattern. The V3 sequence, charge, co-receptor usage, predicted amino acid in position 11, 25 of V3 loop and motif of ten paired isolates are summarized in Table 1.

Sequence analysis

Phylogenetic tree construction: In every case, both the blood and the CSF sequences of each patient were clustered together and grouped with the reference sequence of subtype E (CM240) as shown in Fig 1.

Genetic distances: The genetic distances of the blood and CSF isolates in corresponding patients ranged from 3.2-19.6 (average 6.8). As shown in Table 2, the percent divergences of envelope glycoprotein (gp 120) in blood isolates were significantly different when compared with those of CSF isolates ($p = 0.003$). Similarly, the percent divergences of variable regions V1/V2, V4 and V5 were significantly different between blood and CSF isolates ($p = 0.009, 0.005, 0.001$ respectively). The pairwise distances in V1/V2 regions of CSF sequences were more diverse than in blood sequences; in contrast, V4 and V5 regions

Table 1

The V3 loop sequence of blood, CSF and neurotropic isolates: charge; amino acid at position 11 and 25; motif of V3; SI phenotype and co-receptor usage.

Strain	V3 loop	Charge[a]	SI[b]	Co-receptor usage[c]
BB12	CTRPSKRVR-STRI GPGQ VWYRTEGVDGDIRKAYC	5	-	CXCR4
BC12	CTRPPKGKK-ST-I GPGQ VWYRTEGVDGDIRKAYC	4	-	CXCR4
BB16	CTRPSSNTKTRVTR GPGR VWYRTGEIGGDIRKAHC	6	-	CXCR4, CCR5
BC16	CTRPSSNTRTKVTR GPGR VWYRTGEIGGDIRKAHC	6	-	CXCR4, CCR5
BB27	CTRPFKSIRRRTSI GQGQ VLYRTGDIIGDITKAYC	5	+	CXCR4, CCR5
BC27	CTRP-SSTRKRTSI GQGQ VLYRTEAIIGDIRKAYC	5	-	CXCR4
BB29	CTRPYN-IKTSMTR GPGH VFYRTGDMIGNPGKPYC	4	+	CXCR4
BC29	CTRPYN-IKTSMTR GPGH VFYRTGDMIGNPGKPYC	4	-	CXCR4, CCR5
BB40	CTRPNNNTRISMSR GPGH VYYRTGDIIGDIRKAYC	4	-	CXCR4, CCR5
BC40	CTRPSNNTRISMSR GPGH IYYRTGDIIGDIRKAYC	4	-	CXCR4
BB52	CTRPSNNTRTSITI GPGQ VFYRTGDIIDGIRQAYC	2	+	CXCR4
BC52	CTRPSNNTRTSITI GPGQ VFYRTGDIIGDIRQAYC	2	+	CXCR4,CCR5
BB54	CTRPFKKVRASYRI GPGK VFHNTGSITGDIRKAYC	7	-	CXCR4,CCR5
BC54	CTRPSNNTRTGIHI GPGQ VFYQTGEIIGDIRKAYC	2	-	CCR5
BB68	CTRPSNNIRTSTRI GPGR VFYRTGAITGDIRKAYC	6	-	CCR5
BC68	CTRPSNNIRTSTRI GPGQ VFYKTGAITGDIRKAYC	5	-	CXCR4,CCR5
BB89	CTRPSNNTRTSTSI GPGQ VFYRTGNIIGDIRKAYC	4	-	CXCR4
BC89	CTRPFENIKTRMTM GPGH VFYKTGEITGDIRKAYC	4	-	CCR3[d]
BB95	CTRPYNYTRIRMTT GPGR VFYRTGEIVGDIRKAFC	5	+	CXCR4
BC95	CTRPYNYTRIRMTT GPGR VFYRTGEIVGDIRKAFC	5	-	CXCR4
***	CTRPYN-TKTRMTR GPGH VFYRTGDIIGDIRRAYC			
*	CTRPYN-TKTRMTR GPGH VFYRTGDIIGDIRRAYC (brain signature sequence)			

[a]Positive net charge of amino acid in V3.

[b]Syncytium inducement was observed in MT-2 cell line.

[c]All viruses have been tested for the ability to use CCR5 and CXCR4; the receptors used by each strain are indicated. For both receptors to be listed, the least efficiently used receptor must support virus entry by 10% of the level supported by the most efficiently used receptor.

[d]Ghost transfected CCR3 cell line had been used in order to test CCR3 co-receptor usage.

*** neurotropic sequence derived from neuroblastoma cell line which was infected by blood isolate (BB52 strain).

Amino acids at position 11 and 25 of V3 loop are underlined.

Table 2

Pairwise distances of HIV-1 env (gp120) of blood and CSF isolates.

	% nucleotide distance		
	Blood isolates	CSF isolates	p-value
gp120	22.05	24.09	0.003
V1/V2	17.37	19.44	0.009
V3	20.04	21.41	0.351
V4	21.08	18.44	0.005
V5	14.58	10.99	0.001

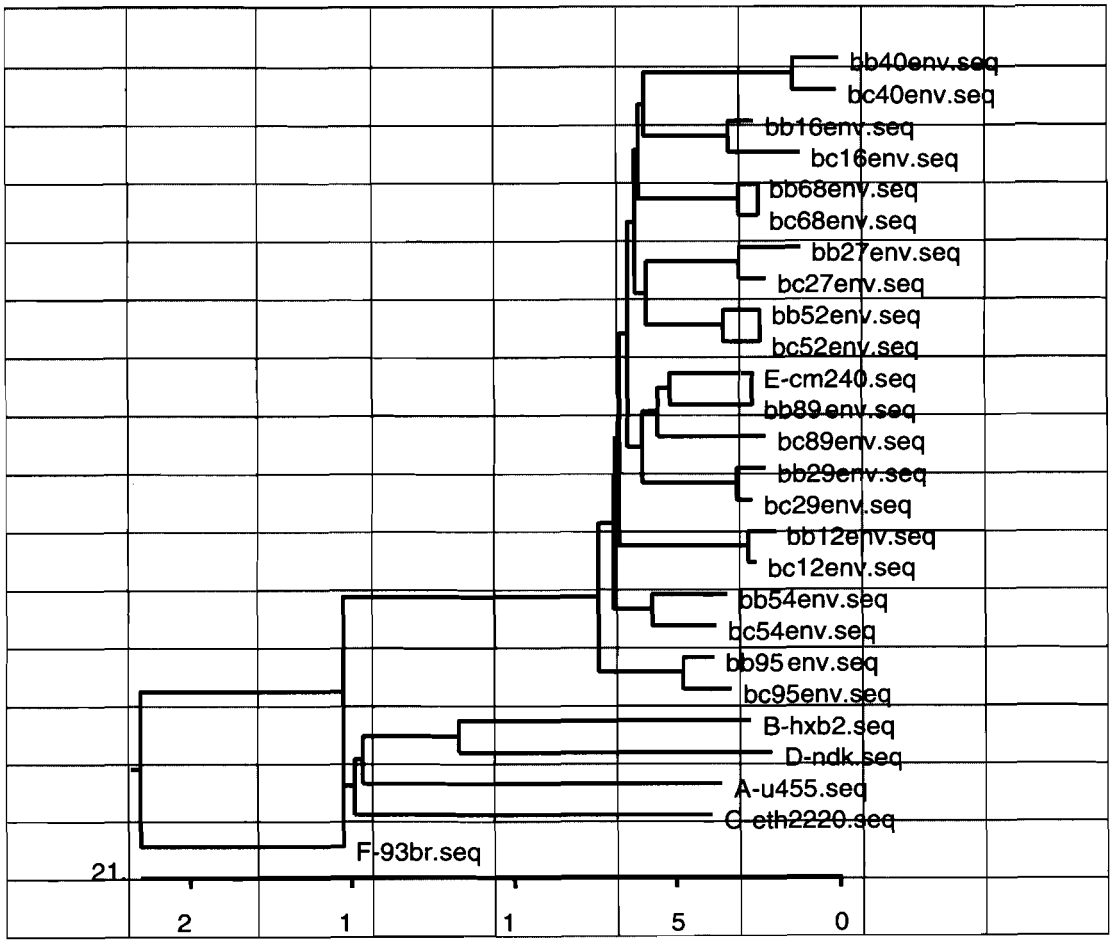

Fig 1–The phylogenetic relationship of paired blood and CSF isolates obtained from direct PCR sequencing. The phylogenetic tree was constructed on the basis of paired-wise differences between nucleotide sequences. All sequences are grouped in subtype E (CM240). Reference sequences of subtype A-F from HIV sequence database were included.

were more conserved in CSF sequences than in blood sequences.

DISCUSSION

Previous study has shown that the rate of HIV isolation from the CSF samples of infected patients with and without neurological symptoms ranges between 40 and 60% (Peeters *et al,* 1995). In the present study, HIV-1 could be cultured from only 13.3% of tested CSF samples. Since most of the samples were contaminated with cryptococcal organisms, culturing HIV-1 from cell-free virus was generally unsuccessful. However, other methodological factors, such as the time of sample processing and patient selection, might also influence the yield of positive culture, as previously reported (Di Stefano *et al,* 1998).

HIV-1 isolates obtained from simultaneous samples of CSF and blood were tested for their cyto pathicity and co-resceptor usage. With regards to the syncytium inducing phenotype, it was confirmed that CSF isolates are less cytopathic than blood isolates. In these syncytium-inducing (SI) sequences, the number of basic amino acids in the V3 region was quite low, ranging from 2 to 5, when compared with previous studies. Moreover, the absence of predicted amino acids (positive charge) at position 11, 18 and 25 of the V3 loop was a feature of most of the sequences. No relationship between positive charge and SI phenotype was observed. These results are due to the fact that the direct sequences of SI isolates were derived from the majority of the HIV-1 population while the SI phenotype represented from both majority and quasispecies. It is likely that regions other than V3 could affect the cytopathicity, a suggestion supported by finding that the syncytium-inducing phenotype was clearly mapped to four amino acids in the V1/V2 regions (Shieh *et al,* 2000).

Considering co-receptor usage, most isolates from AIDS-defining subjects used CXCR4 and CCR5 or CXCR4 as co-receptors. These data are consistent with previous observations: some late stage variants use CXCR4 alone (X4; T-cell tropic) while others use both CXCR4 and CCR5 (R5X4; dual tropic) (Glushakova *et al,* 1999; Shiino *et al,* 2000). Using CXCR4 has advantages for HIV-1 during disease progression. It has been proposed that dual tropic R5X4 strains are intermediates in the evolution from R5 to X4 (Singh and Collman, 2000). In addition, CCR3 co-receptor was also reported to use for HIV-1 entry of many M-tropic and dual tropic HIV-1 isolates (Connor *et al,* 1997) and is involved in entry into as well as infection of microglia. However, CCR3 co-receptor usage was less common in this study: seen in only one CSF isolate (BC89). Mapping the co-receptor domains responsible for HIV-1 entry showed that virus isolates differ significantly in their dependence upon specific regions of the co-receptors. However, the contribution of theV3 through V5 region of the 89.6 dual tropic strain to CCR3 utilization has been demonstrated (Smyth *et al,* 1998): for this reason, it is not solely V3 that is involved in determining co-receptor usage (Kato *et al,* 1999). Other regions of the envelope glycoprotein play additional roles. Potential candidate regions include the V2 (Koito *et al,* 1995) and V4 -V5 regions (Smyth *et al,* 1998) and even the more conserved N-terminal portion of the V3 loop itself (Wang *et al,* 1998). The differences in phenotype and co-receptor usage of paired blood and CSF isolates in intraperson indicate that HIV- 1 may evolve differently in the brain and in the blood. (Di Stefano *et al,* 1998).

Of the patients under study, the mean distance divergence of envelope glycoprotein between blood and CSF isolates was 6.8. The high diversity of paired blood and CSF isolates reflected the long duration of HIV infection (Murphy *et al,* 1993). As reported in a previous study, the V3 region of blood was more diverse than that of CSF isolates (Korber *et al,* 1994). However, in this study, there was no significant difference found in V3 regions of blood and CSF isolates ($p = 0.351$); furthermore, the diversity of the V1/V2 region of CSF isolates was greater than that of blood isolates (significant difference; $p = 0.009$). The

results suggested that evolution of CSF isolates in these regions provided an advantage for HIV-1 in CNS infection. In contrast, the more conserved V4 and V5 regions of CSF isolates implied that these two variable regions were necessary in the process of CNS infection. In addition, these molecular changes might depend upon the number of exposures to the HIV, the stage of the disease and the nature of the initial infecting virus strain (Chang *et al*, 1998).

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REFERENCES

Chang J, Jozwiak R, Wang B, *et al.* Unique HIV type 1 V3 region sequences derived from six different regions of brain: region-specific evolution within host-determined quasispecies. *AIDS Res Hum Retrovir* 1998; 14: 25-30.

Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR. Change in co-receptor use correlates with disease progression in HIV-1-infected individuals. *J Exp Med* 1997; 185: 621-8.

Di Stefano M, Monno L, Fiore JR, *et al.* Neurological disorders during HIV-1 infection correlate with viral load in cerebrospinal fluid but not with virus phenotype. *AIDS* 1998; 12: 737-43.

Edinger AL, Amedee A, Miller K, *et al.* Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains. *Proc Natl Acad Sci USA* 1997; 94: 4005-10.

Gabuzda D, He J, Ohagen A, Vallat AV. Chemokine receptors in HIV-1 infection of the central nervous system. *Semin Immunol* 1998; 10: 203-13.

Glushakova S, Yi Y, Grivel JC, *et al.* Preferential coreceptor utilization and cytopathicity by dual-tropic HIV-1 in human lymphoid tissue *ex vivo*. *J Clin Invest* 1999; 104: R7-R11.

Kato K, Sato H, Takebe Y. Role of naturally occurring basic amino acid substitutions in the human immunodeficiency virus type 1 subtype E envelope V3 loop on viral co-receptor usage and cell tropism. *J Virol* 1999; 73: 5520-6.

Keys B, Karis J, Fadeel B, *et al.* V3 sequences of paired HIV-1 isolates from blood and cerebrospinal fluid cluster according to host and show variation related to the clinical stage of disease. *Virology* 1993; 196: 475-83.

Koito A, Stamatatos L, Cheng-Mayer C. Small amino acid sequence changes within the V2 domain can affect the function of a T-cell line-tropic human immunodeficiency virus type 1 envelope gp120. *Virology* 1995; 206: 878-84.

Korber BT, Kunstman KJ, Patterson BK, *et al.* Genetic differences between blood- and brain-derived viral sequences from human immunodeficiency virus type 1-infected patients: evidence of conserved elements in the V3 region of the envelope protein of brain-derived sequences. *J Virol* 1994; 68: 7467-81.

Lavi E, Kolson DL, Ulrich AM, Fu L, Gonzalez-Scarano F. Chemokine receptors in the human brain and their relationship to HIV infection. *J Neurovirol* 1998; 4:301-11.

Lipton SA, Gendelman HE. Seminars in medicine of the Beth Israel Hospital, Boston. Dementia associated with the acquired immunodeficiency syndrome. *N Engl J Med* 1995; 332: 934-40.

McCutchan FE, Salminen MO, Carr JK, Burke DS. HIV-1 genetic diversity. *AIDS* 1996; 10: S13-20.

Miller RJ, Meucci O. AIDS and the brain: is there a chemokine connection? *Trends Neurosci* 1999; 22: 471-9.

Murphy E, Korber B, Georges-Courbot MC, *et al.* Diversity of V3 region sequences of human immunodeficiency viruses type 1 from the central African Republic. *AIDS Res Hum Retrovir* 1993; 9:997-1006.

Peeters MF, Colebunders RL, Van den Abbeele K, *et al.* Comparison of human immunodeficiency virus biological phenotypes isolated from cerebrospinal fluid and peripheral blood. *J Med Virol* 1995; 47: 92-6.

Shieh JT, Martin J, Baltuch G, Malim MH, Gonzalez-Scarano F. Determinants of syncytium formation in microglia by human immunodeficiency virus type 1: role of the V1/V2 domains. *J Virol* 2000;

74: 693-701.

Shiino T, Kato K, Kodaka N, *et al.* A group of V3 sequences from human immunodeficiency virus type 1 subtype E non-syncytium-inducing, CCR5-using variants are resistant to positive selection pressure. *J Virol* 2000; 74: 1069-78.

Singh A, Besson G, Mobasher A, Collman RG. Patterns of chemokine receptor fusion cofactor utilization by human immunodeficiency virus type 1 variants from the lungs and blood. *J Virol* 1999; 73: 6680-90.

Singh A, Collman RG. Heterogeneous spectrum of coreceptor usage among variants within a dualtropic human immunodeficiency virus type 1 primary-isolate quasispecies. *J Virol* 2000; 74: 10229-35.

Smyth RJ, Yi Y, Singh A, Collman RG. Determinants of entry cofactor utilization and tropism in a dualtropic human immunodeficiency virus type 1 primary isolate. *J Virol* 1998; 72: 4478-84.

Wang WK, Dudek T, Zhao YJ, *et al.* CCR5 co-receptor utilization involves a highly conserved arginine residue of HIV type 1 gp120. *Proc Natl Acad Sci USA* 1998; 95: 5740-5.