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Genetic Composition of Human Immunodeficiency Virus Type 1 in Cerebrospinal Fluid and Blood without Treatment and during Failing Antiretroviral Therapy†

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Human immunodeficiency virus (HIV) infection of the central nervous system (CNS) is a significant cause of morbidity. The requirements for HIV adaptation to the CNS for neuropathogenesis and the value of CSF virus as a surrogate for virus activity in brain parenchyma are not well established. We studied 18 HIV-infected subjects, most with advanced immunodeficiency and some neurocognitive impairment but none with evidence of opportunistic infection or malignancy of the CNS. Clonal sequences of C2-V3 env and population sequences of pol from HIV RNA in cerebrospinal fluid (CSF) and plasma were correlated with clinical and virologic variables. Most (14 of 18) subjects had partitioning of C2-V3 sequences according to compartment, and 9 of 13 subjects with drug resistance exhibited discordant resistance patterns between the two compartments. Regression analyses identified three to seven positions in C2-V3 that discriminated CSF from plasma HIV. The presence of compartmental differences at one or more of the identified positions in C2-V3 was highly associated with the presence of discordant resistance ($P = 0.007$), reflecting the autonomous replication of HIV and the independent evolution of drug resistance in the CNS. Discordance of resistance was associated with severity of neurocognitive deficits ($P = 0.07$), while low nadir CD4 counts were linked both to the severity of neurocognitive deficits and to discordant resistance patterns ($P = 0.05$ and 0.09, respectively). These observations support the study of CSF HIV as an accessible surrogate for HIV virions in the brain, confirm the high frequency of discordant resistance in subjects with advanced disease in the absence of opportunistic infection or malignancy of the CNS, and begin to identify genetic patterns in HIV *env* associated with adaptation to the CNS.

The primate lentiviruses human immunodeficiency virus type 1 (HIV-1) and the simian immunodeficiency virus SIVmac invade the central nervous system (CNS) within days to weeks of primary infection (11, 37, 102), and in the case of HIV result in neurocognitive dysfunction in up to 50% of untreated individuals in late disease (31, 36, 57, 71, 74). Such neurocognitive changes have been linked to postmortem neuropathologic findings of HIV encephalitis (10) and injury to the synaptodendritic apparatus (24, 56). This neuropathology occurs independently of known opportunistic infection (OI) or neurologic malignancy and requires both productive HIV infection and its immunologic sequelae, in particular, the activation and recruitment of macrophages into the CNS (27, 77, 78, 103).

Vigorous but ultimately ineffective host immune responses impel the rapid evolution of HIV-1 in blood and lymphatic tissues (6, 47, 80, 93, 98), but whether similar forces are in play in the CNS is not known. Indeed, the CNS constitutes an immunologically distinct and possibly privileged site that, in the case of other viral pathogens, serves as a chronic sanctuary (12). In addition, HIV predominantly infects microglial cells and perivascular macrophages in the CNS (44, 95), while the principal targets are activated $CD4^+$ lymphocytes in blood and the lymphoid tissues (101). Selective adaptation of replicating HIV populations in the CNS to these cell types might therefore be expected. Thus, findings from earlier studies indicating that HIV populations in the CNS are genetically distinct from virus present in blood or lymphoid tissues (23, 29, 35, 46, 66, 69, 72, 73) are not surprising and may reflect either differences of the principal cellular targets or differences of immune selection. Whether particular sequence motifs define either neurotropism or neurovirulence remains unresolved (29, 46, 66, 73, 79). Observed sequence differences may arise due to genetic drift rather than to increased replicative efficiency or immune selection in the CNS (42).

The potential for differential effects of antiretroviral treatment on CNS and systemic HIV populations both has clinical implications and provides opportunities for the investigation of HIV genetics in the CNS (20, 21, 59, 75, 87, 90). Although many studies using CSF virus as a surrogate for virus in brain parenchyma suggest that highly active antiretroviral therapy (ART) is usually effective over short periods in the CNS (26,

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† Supplemental material for this article may be found at http://jvi.asm.org/.

TABLE 1. Subject characteristics[a]

Patient	Current CD4 (cells/mm^3)	Nadir CD4 (cells/mm^3)	Plasma RNA (log copies/ml)	CSF RNA (log copies/ml)	CSF WBC (cells/mm^3)	NP GDS	Any Rx	Current Rx
A	355	133	4.9	4.5	58	1.28	+	−
B	219	112	5.5	5.2	4	2.13	+	+
C	312	312	5.7	5.2	312	0.44	−	−
D	55	23	5.9	4.0	2	0.69	+	−
E	277	277	4.7	4.0	44	1.41	+	−
F	688	391	4.7	4.8	16	0.88	+	+
H	267	248	4.9	4.3	18	0.53	+	−
I	112	112	6.0	4.6	6	NA	+	−
J	153	153	5.2	6.2	3	0.75	−	−
K	221	221	5.7	4.4	16	0.50	−	−
L	32	10	6.0	3.2	3	0.63	+	+
M	91	91	5.2	3.8	4	0.56	+	+
N	145	145	4.5	4.6	31	0.31	+	+
O	43	30	5.6	3.3	1	1.40	−	−
P	60	56	5.9	3.2	1	1.18	+	−
Q	68	68	5.1	3.5	2	0.94	+	−
R	16	16	5.1	2.9	3	3.50	+	+
S	32	32	6.0	3.2	3	2.88	+	−
Median	128.5	112	5.35	4.15	4	0.88		

[a] NA, not available; any Rx, past or present antiretroviral therapy; current Rx, on antiretroviral therapy at time of sampling or within 2 months of sampling. Subject J had been treated only with zidovudine monotherapy for <2 weeks, more than 2 years prior to enrollment, and was classified as treatment naïve.

33), the long-term durability of these responses remains unaddressed. Differences in baseline HIV genetics, the longevity of infected microglia, and limited drug penetration justify concern for long-term outcomes. Indeed, studies suggest that neurological manifestations of HIV infection constitute an increasing proportion of AIDS-defining illness in some settings (17, 18, 64), and when drug resistance develops, discordant resistance patterns between CNS and blood virus have been observed in many though not all studies of HIV in brain (59, 99) or cerebrospinal fluid (CSF) (14, 15, 84, 91, 94).

In this study, we examined the genetics of HIV-1 in subjects either failing therapy or not on treatment who underwent paired blood and CSF sampling in the absence of opportunistic neurological disease. Population sequences of *pol* and clonal sequences of C2-V3 *env* from these subjects revealed genetically distinct populations in the CSF of most subjects irrespective of antiviral therapy. Analysis by logistic regression identified three to seven codons in C2-V3 that are important as tissue-specific genetic patterns. Subjects harboring these signature patterns in CSF virus were very likely to exhibit discordant patterns of drug resistance in CSF and plasma. These results provide evidence that HIV sequence evolution in the CNS is shaped by compartment-specific selective pressures reflected, in a majority of cases, by genetically discrete CSF HIV populations.

(Presented in part at the 10th Conference on Retroviruses and Opportunistic Infections, Boston, Mass., February 2003, and the Workshop on HIV Genetics and NeuroAIDS, Washington, D.C., December 2002.)

MATERIALS AND METHODS

Subjects. Twenty-one individuals enrolled in longitudinal clinical studies at the HIV Neurobehavioral Research Center between years 1998 and 2002 were initially studied. All subjects had stable or no antiviral therapy for at least 2 months prior to study, had plasma and CSF HIV RNA of >500 copies/ml, and had no evidence of systemic or CNS OIs or malignancy based on clinical, laboratory, and neuro-imaging studies. Data were available on past and present therapy, current HIV RNA and CD4 counts, nadir CD4 counts, and CSF cell counts. All studies were conducted in compliance with local Institutional Review Board guidelines and with subjects' written informed consent.

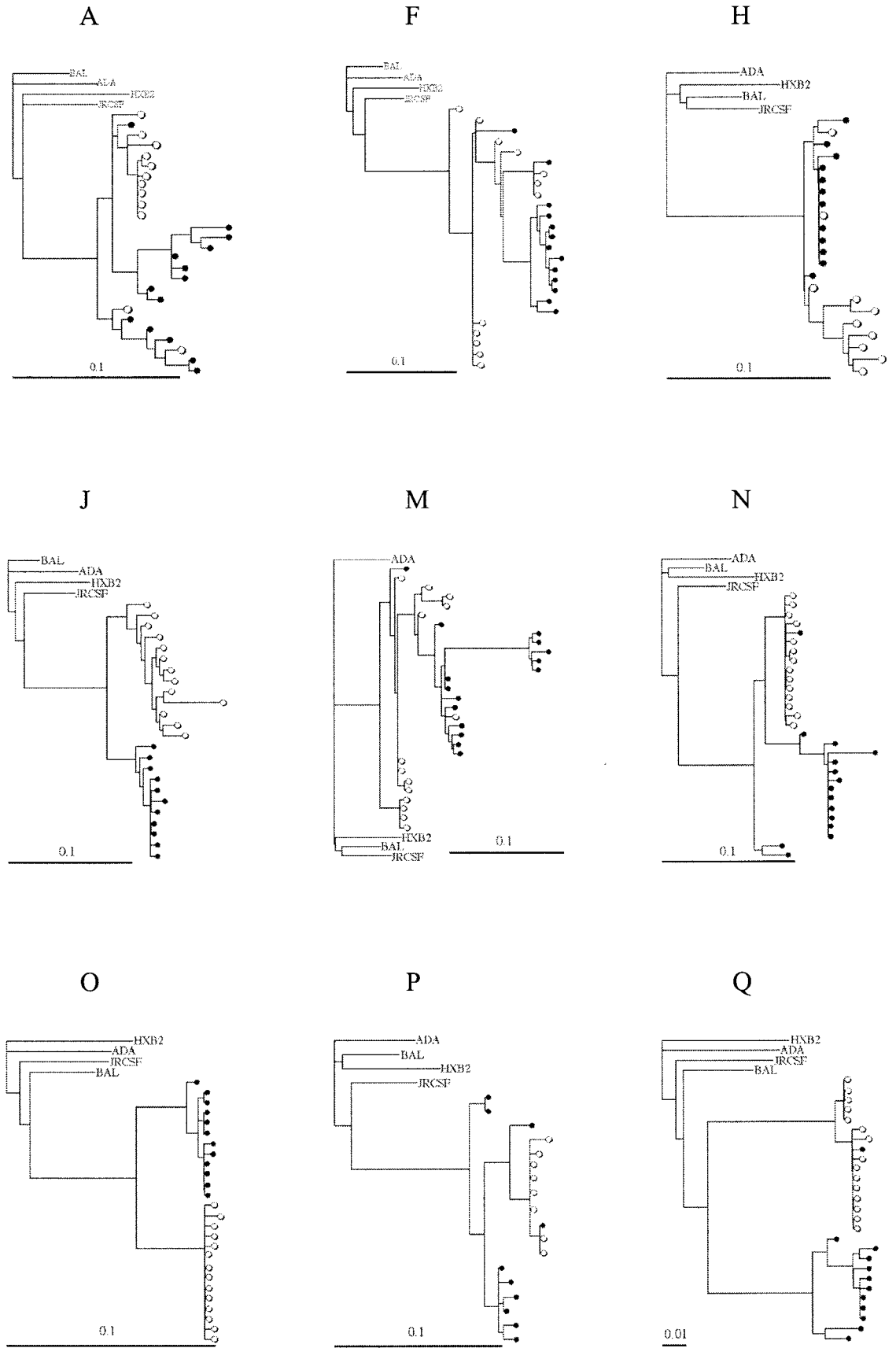
Specimen processing. Paired blood from peripheral venipuncture in acid citrate dextrose tubes and CSF from lumbar punctures were collected (typically collected within 1 h of each other) and processed within 2 h of collection. Plasma and cell-free CSF were aliquoted, frozen, and stored at −70°C until processing. All subsequent plasma and CSF processing was performed separately to minimize the within-subject cross-contamination of samples.

HIV RNA quantitation. Plasma and CSF HIV RNA levels were measured by the Roche Amplicor Monitor assay (lower limit of detection of 400 copies/ml) or the Ultrasensitive assay (lower limit of detection of 50 copies/ml) with modifications as previously described (21).

TABLE 2. Summary of signature residues significantly different between compartments for each subject

Subject	CSF or plasma virus significantly partitioned by Maddison-Slatkin test?[a]	Positions with compartmental difference significant by Fisher exact test
A	Yes	308, 341, 362
F	Yes	304
H	Yes	341
J	Yes	308, (360), 362
M	Yes	308, (309)
N	Yes	304
O	Yes	304
P	Yes	340, 341
Q	Yes	293, 308
R	Yes	360
S	Yes	293, 300, 308, (340), 341
B	(Yes)	340
L	(Yes)	None
C	No	None
D	No	None
E	No	None
K	No	(293)
I	No	308

[a] Parenthetical results indicate $0.01 < P < 0.05$; otherwise $P < 0.01$.


A
BAL
ADA
HXB2
JRCSF
0.1
F
BAL
ADA
HXB2
JRCSF
0.1
H
ADA
HXB2
BAL
JRCSF
0.1
J
BAL
ADA
HXB2
JRCSF
0.1
M
ADA
HXB2
BAL
JRCSF
0.1
N
ADA
BAL
HXB2
JRCSF
0.1
O
HXB2
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BAL
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JRCSF
0.1
Q
HXB2
ADA
JRCSF
BAL
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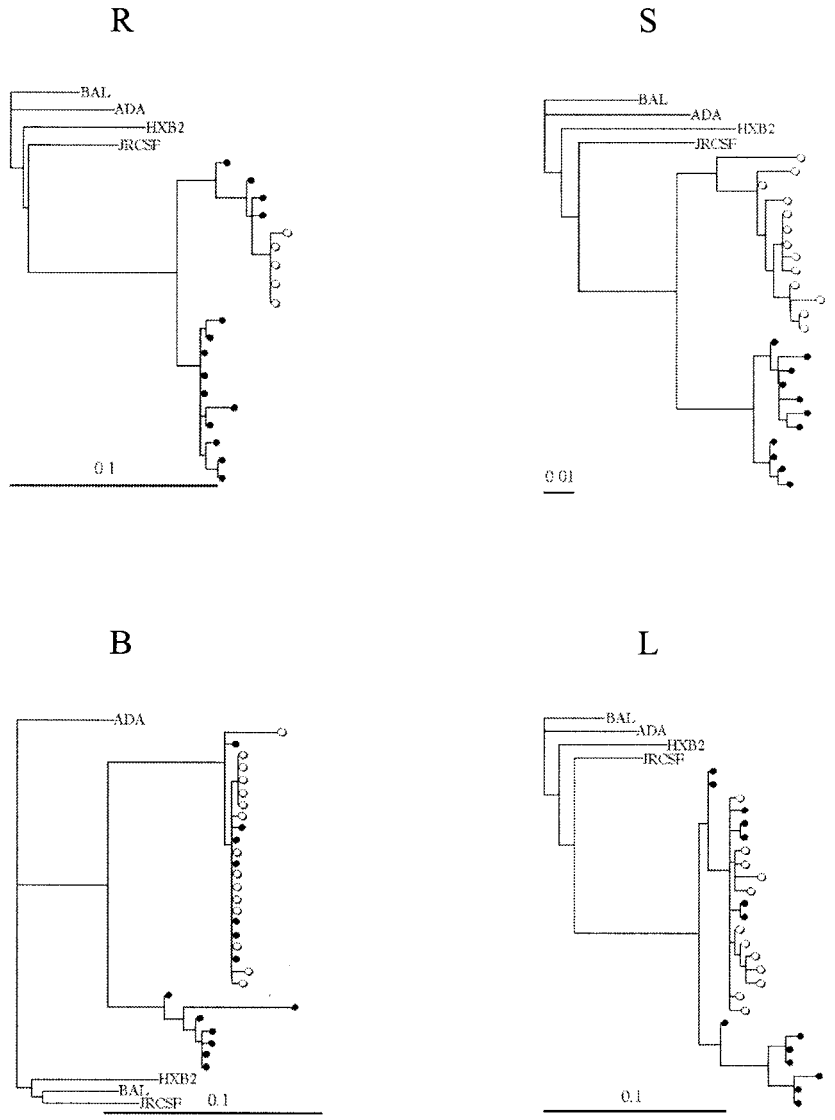

FIG. 1. Phylogenetic reconstruction from C2-V3 env clonal sequences for each subject. Maximum likelihood trees were inferred using DNAML (Phylip 3.6a; J. Felsenstein) with a transition/transversion ratio of 2.0 and a gamma-distributed substitution rate for all clonal sequences for individual subjects and sequences of four prototypic viral strains, HXB2, ADA, BAL, and JRCSF. Sequences from plasma are shown as solid circles, and sequences from CSF are shown as open circles. Trees are grouped according to evidence for significant clustering based on the Maddison-Slatkin test with a P value of <0.01 (A, F, H, J, M, N, O, P, Q, R, and S), $0.01 < P < 0.05$ (B and L), and $P > 0.05$ (C, D, E, I, and K).

NP testing. Neuropsychological (NP) testing was performed within 2 days of CSF sampling for 17 of 18 evaluable subjects except for subject N, whose testing was performed 17 months earlier. Overall NP performance was summarized by calculating global deficit scores (GDS). In brief, the GDS approach quantifies the number and degree of impaired NP test scores across the battery while attaching relatively less significance to performances that are within (or above) normal limits. For each of the NP test variables, raw scores were converted to T-scores (mean, 50; standard deviation, 10) using published demographically corrected normative data. The T-scores were then transformed into deficit scores using the following conversions: >40T = 0; 39T to 35T = 1; 34T to 30T = 2; 29T to 25T = 3; 24T to 20T = 4; and <19T = 5. The deficit scores from each NP test were then averaged to derive a GDS for each participant (range, 0 to 5). Prior research supports the construct validity of the GDS approach as an indicator of global NP functioning in persons with HIV infection and indicates that the GDS is a reliable and valid alternative to clinical ratings and, more importantly, can be generalized across different NP test batteries (9, 36). GDS above 0.5 were interpreted as signifying NP impairment.

Nucleotide sequencing. (i) pol sequencing. pol sequencing was performed using the Viroseq version 2.0 system (Applied Biosystems, Alameda, Calif.) on an ABI 3100 genetic analyzer according to the manufacturer's protocol. In brief, 0.5 to 2.0 ml of cell-free CSF or plasma was thawed and centrifuged at 23,900 × *g* and 4°C for 1 h to pellet virus. We chose to concentrate virus even though most samples contained sufficient HIV copy numbers for assays and successful amplification without further concentration in order to improved representative sampling. RNA was extracted and resuspended in 50 to 100 μl of diluent according to the protocol. A 10-μl aliquot of resuspended RNA was used in pol sequencing; the remaining 40 μl was used for clonal C2-V3 sequence studies. Raw data from pol sequencing were edited and interpreted using Viroseq HIV genotyping system software version 2.5 (Applied Biosystems, Foster City, Calif.) to generate a genotypic drug resistance profile. The default base-caller for this software identifies a minority HIV population when it is present in at least a ~30% proportion.

(ii) env sequencing. Reverse transcription and PCR amplification of C2-V3 env for each sample was performed in triplicate or quadruplicate using the

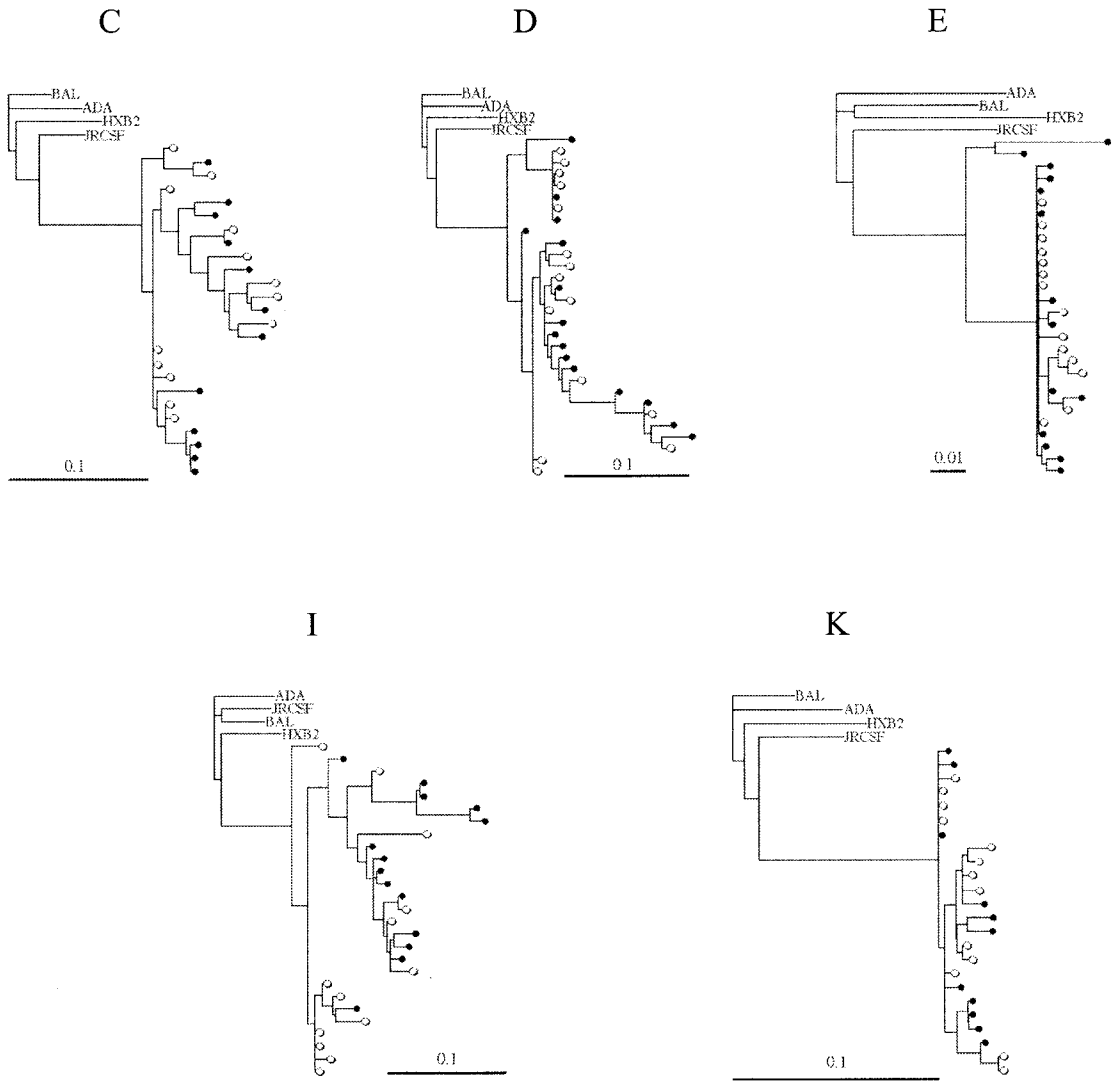

FIG. 1—*Continued.*

Finnzyme one-step reverse transcription-PCR (RT-PCR) kit (MJ Research, Waltham, Mass.) and primers V3Fout and V3Bout as previously described (32) in a 25-μl reaction volume. A 2.5-μl aliquot of first-step RT-PCR product was used in the second, nested PCR with primers V3Fin and V3Bin (32) and Tris (pH 8.0), 20 pmol of each primer, 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, and bovine serum albumin (10 mg/ml) in a total volume of 50 μl. Cycling parameters were 94°C for 2 min; 94°C for 30s, 54°C for 30 s, and 72°C for 1 min for 35 cycles; and 72°C for 10 min. All assays were conducted under conditions to minimize the potential for PCR contamination by utilizing aerosol-resistant pipette tips, dedicated PCR reagents, and laminar flow hoods. All assays included negative controls. Nested PCR products were visualized by agarose gel electrophoresis and ethidium staining. Replicate PCR products were proportionately pooled and cloned using the TOPO-TA cloning system (Invitrogen, Carlsbad, Calif.). Clones were selected by blue-white screening and expanded in 4.0-ml broth cultures. Plasmid clones were purified using QIAGEN Mini-prep kits (QIAGEN). Purified plasmids were sequenced in both directions with −20M13 primer (5′-GTAAAACGACGGCCAG-3′) and Topo forward primer (5′-TGGATATCTGCAGAATTCG-3′) using Prism Dye terminator kits (ABI) on an ABI 3100 genetic analyzer. Sequences were compiled, aligned, and edited using Sequencher 4.0 (Genecodes, Ann Arbor, Mich.) and Clustal (version 1.81).

Genetic analysis. Phylogenetic analysis was performed using DNAML (Phylip 3.6a; J. Felsenstein) (T/T ratio of 2.0, gamma-distributed substitution rate) on clonal sequences for individual subjects and DNADIST, NEIGHBOR, and CONSENSE for 100 bootstrapped data sets from all sequences (Phylip 3.5; J. Felsenstein). Trees were displayed using Treeview (Roderic DM Page). Assessment of degree of intercompartment segregation was performed by testing for panmixis using gene phylogenies (38, 86) as implemented in MacClade (Sinauer, Sunderland, Mass.). In brief, from the maximum likelihood trees for each individual subject's C2-V3 sequences and their characterization according to compartment of origin, the minimum number of intercompartment migration events allowed by the tree was tallied. This result was compared to the distribution of migration events for 1,000 randomly generated trees. Evidence of restricted gene flow (compartmentalization) was documented when <1% (or between 1 and 5%) of the random trees required the same or a fewer number of migration events as for the sample data (65, 86). Assessment of the role of selection on HIV genetic composition was performed using a *Z* test for selection with a modified Nei-Gojobori method and p-distances (63) as implemented in MEGA (49). Tests for positive selection, neutral evolution, and purifying selection were performed at sites identified as important for distinguishing CSF from plasma virus.

Analysis of sequence differences between CSF and plasma virus. Prediction of coreceptor usage was based on composition at residues 11 and 25 of the V3 loop (codons 306 and 322 of HXB2 gp120) (25, 85). Residues at which amino acid (AA) frequencies varied significantly between compartments were identified by Monte Carlo approximation of the Mantel-Haenszel and likelihood ratio tests, based on identity of individual AA or on chemical class of AA (positive, negative, neutral, or hydrophobic), with a *P* value of <0.05 considered significant. Posi-

tions with intercompartmental variation by both of these univariate measures were included in a stepwise logistic regression (with entry cutoff P value of <0.10 and retention cutoff P value of <0.05). Forward logistic regression was performed with the entry criterion P value of <0.05. Analyses were performed on the entire data set as well as on "pruned" data, including only those subjects exhibiting phylogenetic segregation of CSF and plasma sequences verified to be significant by the Maddison-Slatkin test ($P < 0.01$) (86).

Statistical analysis of group characteristics. Fisher's exact test was used to compare proportions between groups. In some cases as noted, parameters were logistically fitted and a whole-model test of significance was applied. Statistical analyses were performed using SAS V8 and JMP IN (SAS Institute, Cary, N.C.).

Nucleotide sequence accession number. The pol and env sequences have been to GenBank and assigned accession numbers AY859056 to AY859091.

RESULTS

PCR amplification for both C2-V3 env and resistance genotyping was successful in 18 of 21 subjects but failed in 3 subjects. HIV RNA concentrations did not appear to predict failure for PCR amplification of either pol or env sequences. The median levels of HIV RNA ($\log_{10}$ copies per milliliter) for subjects whose amplifications failed versus succeeded were similar in plasma (5.29 versus 5.35) and CSF (4.08 versus 4.15). Subject characteristics, treatment history, and NP test scores for the remaining 18 subjects are shown in Table 1. Subjects had relatively advanced disease (median CD4, 128 cells/mm^3) and the majority (13 of 18) were neurocognitively impaired as defined by a GDS of >0.5.

C2-V3 env sequences. Most subjects (13 of 18) exhibited partitioning of CSF from plasma C2-V3 sequences (Fig. 1). For 11 subjects, the Maddison-Slatkin test for compartment-based partitioning was highly significant ($P < 0.01$), and all exhibited clear segregation of the majority of the CSF from plasma sequences on visual inspection of phylogenetic trees. Two other subjects had evidence of partitioning ($0.01 < P < 0.05$) but appeared to have a greater degree of intermingling of CSF and plasma sequences with only subsets of sequences that were segregated. In 17 of 18 subjects, sequences from CSF and plasma from the same subject clustered more closely to one another than did either CSF-CSF or plasma-plasma sequences between subjects, reflecting the impact of common ancestry of within-subject virus populations on the phylogenetic reconstruction (Fig. 2). In subject Q, the sequences formed two divergent but related clusters. While these sequences were more closely related to one another than to sequences from other subjects, the bootstrap value was marginal (43). Sequences from subject B comprised two very divergent, seemingly unrelated clusters, one having both CSF and plasma sequences and one with only plasma sequences (Fig. 1 and 2). Genetic distance between sequences from these two clusters was approximately 10%. These sequences did not appear to cluster with sequences from any other subjects or with lab strains. Of note, pol sequences from plasma virus in this subject also demonstrated genotypic mixtures at ratios of approximately 50:50 based on peak heights of chromatograms (data not shown). These two cases appear consistent with cocirculation of markedly divergent HIV variants reflecting either replication under very different selective environments or, in the case of subject B, coinfection or superinfection with a second HIV variant (2, 40, 43).

Analysis of differences in env sequence between CSF and plasma virus. Predicted coreceptor usage did not distinguish CSF from plasma viruses in the clonal sequences examined for most subjects. With few exceptions the majority of clones from both CSF and plasma were predicted to be from virus using CCR5 rather than CXCR4, based on the composition of residues 11 and 25 of the V3 loop (codons 306 and 322, respectively, of env) (25, 85) (Fig. 3). Basic residues at position 11 or 25 were found in 3 of 14 plasma and 1 of 13 CSF clones from subject I, 5 of 15 plasma and 0 of 14 CSF clones from subject M, and 11 of 15 plasma and 0 of 14 CSF clones from subject N. Notably, all CSF clones from subject O exhibited an arginine at position 25, but this position was deleted from all 10 plasma clones sequenced. Thus, while we encountered clones predicted to utilize CXCR4 more frequently in plasma virus, this type occurred in only a small number of subjects. CXCR4 using HIV was found occasionally in CSF, consistent with recent reports of its identification from brain parenchyma (66).

Regression analyses were used to verify positions that distinguished CSF from plasma clones for each subject and for all the sequenced clones. Residues 293, 308, and 341 were associated with CSF compartmentalization in the entire sequence

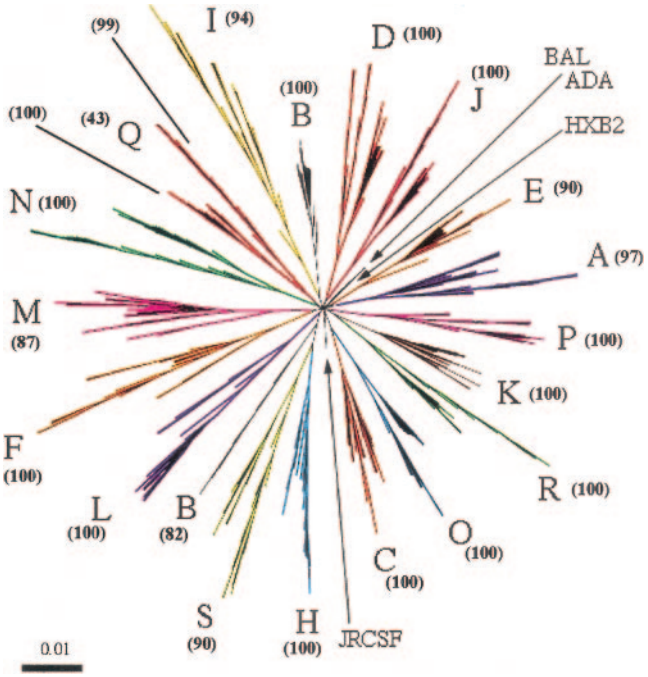

FIG. 2. Neighbor-joining tree from all subjects, demonstrating separate sequence clusters for each subject. The result shown is a consensus tree from 100 bootstrapped data sets. The branches for each subject's sequences were assigned a random color for identification purposes, and the letters identify subject source for each sequence cluster. Numbers shown in parentheses represent bootstrap values supporting branching order defining individual subject clusters. Not all significant bootstrap values could be shown at this resolution. In all cases except subjects B and Q, intrasubject sequences formed discrete clusters with high bootstrap values. Sequences from subject B formed two discrete clusters, each with high bootstrap values, one containing only sequences from plasma and one with a mixture of plasma and CSF clones. Neither cluster intermingled with sequences from other subjects or with laboratory strains. Subject Q sequences clustered with only a modest bootstrap value of 43. However, each of the two subsets of sequences from subject Q were supported by high bootstrap values (shown) as were those for several other subjects (not shown).

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
A-10C	-	-	-	S	H	A	-	-	V	X4
A-01C	-	-	-	S	P	A	-	-	V	
A-04C	-	-	-	S	H	A	-	-	V	
A-05C	-	-	-	S	H	A	S	-	V	
A-07C	-	-	-	S	S	A	-	-	V	
A-08C	-	-	-	S	H	A	-	-	V	
A-09C	-	-	-	S	H	A	-	-	V	
A-14C	-	S	-	S	S	A	-	-	-	
A-12C	-	-	-	S	H	A	-	-	A	
A-01P	-	-	-	S	S	A	-	-	V	
A-13P	-	-	-	S	S	G	D	-	D	
A-08P	-	-	-	S	S	A	-	-	N	
A-09P	-	-	-	S	S	A	-	-	V	
A-02P	-	-	-	S	S	A	D	-	D	
A-04P	-	-	-	S	S	A	D	-	H	
A-05P	-	-	-	S	S	A	D	-	D	
A-06P	-	-	-	S	P	A	-	-	V	
A-10P	-	-	-	S	S	A	-	-	N	
A-15P	-	-	-	S	S	A	-	-	N	
A-11P	-	-	-	S	S	A	-	-	N	
A-12P	-	-	-	S	S	A	-	-	V	
A-03P	-	-	-	S	S	A	D	-	D	
A-14P	-	-	-	S	S	A	-	-	N	
B-02C	V	S	-	S	S	S	-	-	V	X7
B-10C	V	S	-	S	S	S	-	-	V	X5
B-06C	V	S	-	S	S	G	-	-	V	
B-07C	V	S	-	S	S	S	-	-	V	
B-01C	V	S	-	S	S	S	-	I	V	
B-04P	V	S	-	S	S	S	-	-	V	X5
B-02P	V	-	-	S	T	A	-	-	-	X3
B-01P	V	S	-	S	S	G	-	-	V	
B-13P	V	S	-	S	T	A	-	-	-	
B-14P	V	S	-	S	S	S	-	-	V	
B-03P	V	-	-	S	T	A	-	-	-	
B-15P	V	-	-	S	T	A	-	-	-	
B-08P	Q	-	-	S	T	D	-	-	-	

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
C-15C	T	-	-	S	P	A	A	-	V	X2
C-11C	T	-	-	S	P	A	A	-	V	
C-10C	T	-	-	?	P	A	A	-	V	
C-14C	T	-	-	S	P	A	A	-	V	
C-01C	T	-	-	S	P	D	A	-	V	
C-08C	T	-	-	S	P	A	A	-	V	
C-06C	T	-	-	S	P	A	A	-	V	
C-12C	T	-	-	S	P	D	A	-	V	
C-13C	A	-	-	S	P	A	A	-	-	
C-09C	T	-	-	S	H	D	A	-	V	
C-15C	T	-	-	S	P	D	A	-	V	
C-02C	T	-	-	S	P	D	A	-	F	
C-02P	T	-	-	S	P	A	D	-	-	X3
C-10P	T	-	-	S	P	A	A	-	-	
C-03P	T	-	-	S	P	D	A	-	V	
C-12P	T	-	-	S	P	A	A	-	-	
C-13P	T	-	-	S	P	A	D	-	V	
C-08P	A	-	-	S	P	D	A	-	V	
C-14P	A	-	-	S	P	A	A	-	-	
C-04P	T	-	-	S	P	D	A	-	V	
C-05P	T	-	-	S	P	D	A	-	V	
C-06P	P	-	-	S	P	A	A	-	-	
D-08C	I	-	-	G	N	E	-	-	A	X2
D-03C	I	-	-	G	N	E	-	-	A	X2
D-09C	I	-	-	G	N	E	-	-	A	
D-04C	I	-	-	G	H	Q	-	-	R	
D-02C	I	-	-	G	H	Q	-	-	R	
D-01C	I	-	-	G	H	Q	-	-	R	
D-10C	I	-	-	G	N	E	-	-	A	
D-11C	I	-	-	G	H	Q	S	-	R	
D-12C	I	-	-	G	H	Q	S	-	R	
D-13C	I	-	-	G	H	Q	-	-	R	
D-14C	I	-	-	G	H	Q	-	-	R	
D-15C	I	-	-	G	N	E	-	-	-	
D-07C	I	-	-	G	N	E	-	-	A	

FIG. 3. Predicted protein sequences at informative sites. All letter designations for AA conform to the standard International Union of Pure and Applied Chemistry code. "." indicates a codon deletion; "-" indicates an AA identical to the AA of HXB2; "≪" indicates a 2-base deletion with frameshift; "?" indicates an indeterminate AA. The HXB2 and JRCSF sequences (Los Alamos National Laboratory) are given at the top of each set of subject sequences. Numbering is according to the HXB2 reference sequence. Codons 300, 304, 306, 308, and 322 are contained within the V3 loop (highlighted with gray background). Residues 306 and 322 (codons 11 and 25 of the V3 loop of clade B consensus) contribute to coreceptor preference (25, 85). Single underlined codon positions delineate positions predictive of compartment of origin when examining the pruned data set only, while double-underlined codon positions denote residues significantly predictive of compartment of origin either with the pruned or the entire data set. Numbers at the end of each row indicate the number of clones exhibiting each pattern of codons. Clonal patterns are displayed individually even if they are identical at these nine codons if they differed at other AA positions not shown (refer to Fig. S1 in the supplemental material for complete AA sequences).

set (data not shown). The set of unique sequences in the subset of subjects exhibiting clear partitioning of CSF and plasma sequences ($n = 11$, pruned subset) were separately analyzed. The P value for evidence of partitioning was <0.01 by both χ^2 analysis and stepwise logistic regression (Table 2; Fig. 4a). Categorization of amino acids by charge and hydrophobicity (as positive, negative, neutral, or hydrophobic) improved the predictive value of positions 293, 308, and 341 (Table 3; Fig. 4b).

In order to assess whether the sequence differences between plasma and CSF virus were due to genetic drift of isolated HIV populations or resulted from selection, a Z-test using a modified Nei-Gojobori method (p distances) for comparing synonymous and nonsynonymous substitutions at codons 293, 308, and 341 was performed for all CSF sequences and for all plasma sequences. For both CSF and plasma virus, the P values strongly supported positive selection at these sites ($P < 0.001$ for both). When all seven sites were included, the P value for positive selection for CSF virus increased to 0.07.

Drug resistance. Fourteen of 18 subjects exhibited mutations conferring drug resistance (70), 8 of 9 receiving antiretrovirals and 6 of 9 not on treatment at the time of study (Table 4). Among the eight subjects with drug resistance on ART, five had discordance in resistance patterns between CSF and plasma HIV. Similarly, three of four subjects with past but not current ART had discordance in resistance patterns between CSF and plasma HIV. Two subjects without a known history of drug treatment exhibited drug resistance. Overall, 50% of subjects demonstrated discordant resistance mutations, a proportion comparable to that in previous reports (14, 91).

Twelve of 14 subjects with resistance had mutations associated with resistance to more than one class of antiretroviral drug. Phylogenetic reconstruction of pol sequences showed that CSF and plasma sequences from the same subject were

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
D-10P	I	-	-	G	H	Q	-	-	R	X2
D-13P	I	-	-	G	N	E	-	-	A	X2
D-02P	I	-	-	G	H	Q	S	-	R	
D-03P	I	-	-	G	N	Q	S	-	R	
D-04P	I	-	-	G	N	E	-	-	A	
D-07P	I	-	-	G	N	E	-	-	A	
D-12P	I	-	-	G	N	E	-	-	A	
D-14P	I	-	-	G	N	E	-	-	A	
D-01P	I	-	-	G	H	Q	-	-	R	
D-06P	I	-	-	G	H	Q	S	-	R	
D-08P	I	-	-	G	N	E	-	-	A	
D-05P	I	-	-	G	N	E	-	-	A	
D-09P	I	-	-	G	H	Q	S	-	A	
E-01C	-	S	-	S	H	A	-	-	-	X11
E-05C	-	S	-	S	H	A	-	-	-	
E-02C	-	S	-	S	H	A	-	-	-	
E-06C	-	S	-	S	H	A	-	-	-	
E-04P	-	S	-	S	H	A	-	-	-	X7
E-03P	-	S	-	S	H	A	-	-	V	
E-08P	-	S	-	S	H	A	-	-	-	
E-09P	-	S	-	S	H	A	-	-	-	
E-01P	-	S	-	S	H	A	-	-	-	
E-12P	-	S	-	S	H	I	-	-	-	
E-02P	-	S	-	S	P	A	-	-	-	
E-06P	-	S	-	S	P	A	-	-	-	
H-03C	K	G	-	S	T	D	-	-	A	
H-12C	K	G	-	S	T	D	D	-	A	
H-13C	K	G	-	S	T	D	D	-	A	
H-06C	K	G	-	S	T	E	-	-	A	
H-09C	K	G	-	S	T	D	D	-	D	
H-02C	K	G	-	S	T	D	D	-	A	
H-08C	K	G	-	S	T	D	D	-	A	
H-14C	K	G	-	S	T	D	-	-	A	
H-01C	-	G	-	S	T	D	D	-	D	
H-15C	K	G	-	S	T	D	-	-	A	
H-07P	K	G	-	S	T	D	D	-	A	X6
H-04P	K	G	-	S	T	D	D	-	A	
H-05P	K	G	-	S	T	D	D	-	A	
H-10P	K	G	-	S	T	.	D	-	A	
H-02P	K	G	-	S	T	D	D	-	A	
H-06P	K	G	-	S	T	D	D	-	A	
H-01P	K	G	-	S	T	D	D	-	A	

	293	300	304	306	308	322	341	360	362
HXB2	E	N	R	R	R	K	N	T	I
JRCSF	K	S	-	S	H	E	-	-	V
F-03C	-	-	-	S	H	S	-	-	-
F-07C	-	-	-	S	H	D	-	-	-
F-09C	-	-	-	S	H	D	-	-	-
F-15C	-	-	-	S	H	D	-	-	-
F-13C	-	-	-	S	H	D	-	-	-
F-08C	-	-	-	S	H	D	-	-	-
F-14C	-	-	-	S	H	D	-	-	-
F-02C	-	-	M	S	H	S	-	-	-
F-11C	-	-	-	S	H	D	-	-	-
F-01C	-	-	M	S	H	S	-	-	-
F-04C	-	-	M	S	H	S	-	-	-
F-06C	-	-	M	S	H	S	-	-	-
F-08P	-	-	M	S	-	S	-	-	-
F-14P	-	-	M	S	-	S	-	-	-
F-06P	-	-	M	S	-	S	-	-	-
F-07P	-	-	M	S	-	I	-	-	-
F-12P	-	-	M	S	-	S	-	-	-
F-13P	-	-	-	S	H	D	-	-	-
F-05P	-	-	M	S	-	S	-	-	-
F-10P	-	-	M	S	H	S	-	-	-
F-11P	-	-	M	S	-	S	-	-	-
F-02P	-	-	M	S	-	S	-	-	-
F-04P	-	-	M	S	-	S	-	-	-
F-15P	-	-	M	S	-	S	-	-	-
F-09P	-	-	M	S	-	S	-	-	-

FIG. 3—*Continued.*

most closely related to one another, which excluded contaminating sequences as an explanation for discordant resistance patterns (data not shown).

Of the nine subjects with discordant drug resistance, resistance mutations were noted in plasma but not in CSF for eight, while only one subject (A) exhibited more resistance in virus from CSF than from plasma. This pattern suggests lesser selective pressure by ART or a smaller population of preexisting HIV with mutations for resistance in the CNS compartment. In several cases of concordant multidrug resistance, the patterns of resistance were extremely complex but were nonetheless identical between CSF and plasma virus, arguing for the emergence of resistance as single rather than parallel events in these cases.

Poor penetration of individual antiviral drugs into the CNS could favor the development of discordant resistance for some drugs. We could not systematically address this issue, since the study subjects were treated with a variety of different antiretroviral regimens. Nevertheless, the frequency of discordance for resistance mutations to nucleoside reverse transcriptase inhibitors, a class with good CNS penetration (26, 82), was no less than for resistance mutations to protease inhibitors, which generally penetrate poorly (3, 28, 50, 52) (Table 4).

Correlates of drug resistance patterns and partitioning of C2-V3 sequence. In subjects with drug-resistant virus, concordant resistance patterns were associated with lack of partitioning between CSF and plasma env sequences, reflecting similar HIV populations in both compartments ($P = 0.035$) (Table 5). This observation is compatible with selection of drug resistance in the HIV population in one compartment, which then spreads to the other perhaps because of mixing of the two populations. Conversely, discordance of resistance patterns correlated with envelope partitioning ($P = 0.035$) and with the presence of differences at signature positions in env as summarized in Table 2 ($P = 0.007$), consistent with the independent evolution of resistance in segregated and autonomously replicating HIV populations. Discordant resistance tended to be more common in subjects with lower nadir CD4 counts, as suggested by Cunningham and colleagues (14).

Treatment history tended to correlate with segregation of CSF and plasma env sequence clusters ($P = 0.09$) (Table 5),

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
I-04C	-	-	-	S	P	Q	-	-	A	X3
I-11C	-	-	-	S	P	Q	-	-	A	
I-10C	-	-	-	S	P	Q	-	-	A	
I-07C	-	-	-	S	S	Q	E	-	A	
I-12C	-	-	-	S	S	Q	-	-	A	
I-13C	-	-	-	S	P	Q	-	-	A	
I-03C	-	-	-	G	H	E	G	I	V	
I-02C	K	-	-	G	H	-	-	-	V	
I-08C	T	-	I	G	H	Q	E	-	A	
I-06C	T	-	I	G	H	Q	E	-	A	
I-01C	T	-	I	G	H	Q	E	-	A	
I-13P	-	-	-	S	S	Q	E	-	P	X2
I-07P	T	-	I	G	H	Q	E	-	A	
I-09P	-	-	-	S	P	Q	E	-	A	
I-11P	-	-	I	G	H	Q	E	-	A	
I-14P	-	-	-	-	S	Q	-	-	A	
I-15P	-	-	I	G	H	Q	E	-	A	
I-10P	-	-	I	G	H	Q	E	-	A	
I-02P	-	-	I	G	H	Q	E	-	A	
I-04P	-	-	-	-	S	Q	-	P	P	
I-06P	-	-	-	-	S	Q	-	-	P	
I-12P	T	-	I	G	H	Q	E	-	A	
I-01P	T	-	I	G	H	Q	E	-	A	
I-05P	T	-	I	G	H	Q	E	-	A	
J-03C	K	-	-	S	P	A	-	R	V	
J-13C	K	-	-	S	P	E	-	R	V	
J-07C	K	-	-	S	P	A	-	R	V	
J-05C	K	-	-	G	P	E	-	R	V	
J-11C	K	-	-	G	P	E	-	-	V	
J-02C	K	-	-	S	P	A	-	-	V	
J-15C	K	-	-	S	P	A	-	-	V	
J-14C	K	-	-	G	P	E	-	S	V	
J-04C	K	-	-	S	P	A	-	R	V	
J-06C	K	-	-	S	P	E	-	R	V	
J-01C	K	-	-	S	P	Q	-	K	V	
J-09C	K	-	-	S	P	A	-	-	V	
J-12C	K	-	-	S	P	A	-	R	V	
J-06P	K	-	-	G	H	E	-	-	N	X4
J-12P	K	-	-	G	H	E	-	-	N	
J-04P	N	-	-	G	H	E	-	-	N	
J-11P	K	-	-	G	H	E	-	-	N	
J-01P	K	-	-	G	H	E	-	-	N	
J-02P	K	-	-	G	H	E	-	-	N	
J-03P	K	-	-	G	H	E	-	-	N	
J-05P	K	-	-	G	H	E	-	-	N	

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
K-04C	A	-	-	S	H	D	D	K	S	X2
K-02C	V	-	-	S	H	D	A	K	-	X2
K-08C	A	-	-	S	H	D	D	K	-	X2
K-01C	A	-	-	S	H	D	D	K	-	
K-14C	A	-	-	S	H	D	D	K	-	
K-06C	A	-	-	S	H	D	D	K	-	
K-07C	A	-	-	S	H	D	D	K	-	
K-11C	A	-	-	S	H	D	D	K	-	
K-03C	A	-	-	S	H	G	D	K	-	
K-10C	A	-	-	S	H	D	D	K	S	
K-04P	T	-	-	S	H	D	D	K	-	X2
K-05P	A	-	-	S	H	D	D	K	-	
K-08P	A	-	-	S	H	D	D	K	-	
K-06P	A	-	-	S	H	D	D	K	-	
K-07P	T	-	-	S	H	D	D	K	-	
K-11P	A	-	-	S	H	D	D	K	-	
K-09P	?	-	-	S	H	D	D	K	-	
K-02P	A	-	-	S	H	D	D	K	-	
K-12P	V	-	-	S	H	D	A	K	-	
K-01P	A	-	-	S	H	D	D	K	-	
L-02C	N	-	-	S	H	E	-	-	A	X4
L-11C	N	-	-	S	H	E	-	-	A	X2
L-15C	N	-	-	S	H	E	-	-	A	
L-13C	N	-	-	S	H	E	-	-	A	
L-10C	N	-	-	S	H	E	-	-	A	
L-12C	N	-	-	S	H	E	-	-	A	
L-04C	N	-	-	S	H	E	-	-	A	
L-01C	N	-	-	S	H	E	-	-	A	
L-14P	N	-	-	S	H	E	-	-	A	X2
L-04P	N	-	-	S	H	A	D	-	A	X2
L-09P	N	-	-	S	H	A	-	-	A	X2
L-01P	N	-	-	S	H	E	-	-	A	X2
L-06P	N	-	-	S	H	E	-	-	A	X2
L-05P	N	-	-	S	H	E	-	-	A	
L-03P	N	-	-	S	H	A	D	-	A	
L-12P	N	-	-	S	H	A	-	-	A	
L-10P	N	-	-	S	H	A	-	-	A	

FIG. 3—*Continued.*

suggesting an effect of treatment on the composition of HIV env genotypes; however, there were relatively few subjects who were drug naïve ($n = 5$) (Table 1). The apparent association of treatment and env sequence partitioning may have reflected a prestudy bias towards treating subjects with more advanced disease. In this small study population, CSF pleiocytosis was not significantly associated with resistance patterns or with the observed partitioning of C2-V3 sequences (data not shown).

Correlates of neurocognitive dysfunction. Nadir but not current CD4 count was correlated with severity of neurocognitive impairment ($P = 0.05$) (Table 5). A whole-model test for the logistic fit of nadir CD4 and presence or absence of neurocognitive impairment also demonstrated a significant association ($P = 0.04$). These correlations are consistent with the increasing prevalence of neurocognitive dysfunction with advancing disease. Of note, partitioning of blood and CSF C2-V3 sequences was not significantly associated with neurocognitive abnormalities. However, subjects with concordant resistance or no resistance had a lower GDS than those with discordant resistance (median GDS = 0.8 for concordant or no resistance, median GDS = 1.6 for discordant resistance; $P = 0.07$). Thus, more advanced disease and the presence of discordant resistance were positively associated with the presence and severity of neurocognitive dysfunction.

DISCUSSION

Infection of the CNS is an important contributor to the morbidity of HIV disease, but study of the in vivo biology of CNS infection by HIV is complicated by the relative inaccessibility of tissues of the CNS. Because of the impracticality of sampling the brain for the antemortem study of HIV genetics, many investigators have examined virus in CSF as a surrogate marker for parenchymal disease (8, 22, 55, 58, 76, 87). Whether HIV RNA in CSF accurately reflects the predominant HIV species replicating in brain parenchyma is unknown, but HIV RNA concentrations in brain and CSF appear to be correlated (58, 96) and the predictive value of CSF HIV RNA but not HIV RNA in blood for neurocognitive impairment suggests a significant relationship (8, 21, 58). In addition to the relative ease of

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
M-12C	-	-	-	S	H	D	-	-	V	X2
M-10C	-	-	-	S	H	D	-	-	V	X2
M-07C	-	-	-	S	H	D	-	-	V	X2
M-05C	-	-	-	S	H	D	-	-	V	
M-01C	-	-	-	S	H	D	-	-	A	
M-03C	-	-	-	S	H	D	-	-	V	
M-08C	-	-	-	S	H	D	-	-	A	
M-02C	-	-	-	S	H	D	-	-	V	
M-04C	-	-	-	S	H	D	-	-	A	
M-15C	-	-	-	S	H	D	-	-	A	
M-11C	-	-	-	S	H	D	-	-	V	
M-07P	-	-	-	S	T	D	-	-	A	X2
M-06P	-	-	-	S	T	D	-	-	V	
M-01P	-	-	-	S	T	D	-	-	A	
M-04P	-	-	-	S	T	D	-	-	A	
M-09P	-	-	-	S	H	D	-	-	«	
M-02P	-	-	-	S	T	D	-	-	A	
M-08P	-	-	K	-	I	D	-	-	A	
M-03P	-	-	K	-	I	D	-	-	V	
M-15P	-	-	K	-	I	D	-	-	V	
M-10P	-	-	K	-	I	D	-	-	V	
M-13P	-	-	K	-	I	D	-	-	A	
M-14P	-	-	-	S	T	D	-	-	A	
M-12P	-	-	-	S	T	D	-	-	V	
M-11P	-	-	-	S	T	D	-	-	A	
N-01C	-	G	-	G	H	E	-	-	V	X6
N-02C	-	G	-	G	H	E	-	-	V	
N-08C	-	G	-	G	H	E	-	-	V	
N-11C	-	G	-	G	H	E	-	-	V	
N-14C	-	G	-	G	H	E	-	-	V	
N-10C	-	G	-	G	H	E	-	-	V	
N-09C	-	G	-	G	H	E	-	-	V	
N-03C	-	G	-	G	H	E	-	-	V	
N-06C	-	G	-	G	H	E	-	-	V	
N-01P	-	G	S	S	H	R	-	-	V	X8
N-11P	-	G	S	S	H	R	-	-	V	
N-04P	-	G	S	S	H	E	-	-	V	
N-02P	-	G	-	S	N	N	-	-	V	
N-03P	-	G	-	S	N	N	-	-	V	
N-06P	-	G	-	G	H	E	-	-	V	
N-09P	-	G	S	S	H	R	-	-	V	
N-15P	-	G	S	S	H	R	-	-	V	

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
O-01C	-	S	N	S	N	R	S	-	V	X10
O-11C	-	S	N	S	N	R	S	-	V	
O-09C	-	S	N	S	N	R	S	-	V	
O-10C	-	S	N	S	N	R	S	-	V	
O-13C	-	S	N	S	N	R	S	-	V	
O-02P	-	-	K	S	N	.	S	-	V	X6
O-03P	-	-	K	S	N	.	S	-	V	X2
O-10P	-	-	K	S	N	.	S	-	V	
O-09P	-	-	K	S	N	.	S	-	V	
P-04C	A	-	-	G	H	A	-	-	V	X4
P-05C	A	-	-	G	H	A	-	-	V	X2
P-03C	A	-	-	G	H	A	-	-	V	
P-11C	A	-	-	G	H	A	-	-	V	
P-07P	A	-	-	G	H	A	K	-	V	X3
P-06P	A	-	-	S	H	D	K	-	V	X2
P-08P	A	-	-	G	H	A	K	-	V	
P-03P	A	-	-	G	H	A	K	-	V	
P-13P	A	-	-	G	H	A	K	-	V	
P-04P	A	-	-	G	H	A	-	-	V	
P-15P	A	-	-	G	H	A	K	-	-	
Q-01C	V	-	-	S	P	E	-	-	V	X9
Q-03C	A	-	-	S	P	E	-	-	V	X5
Q-12C	V	-	-	S	P	E	-	-	V	
Q-01P	-	-	-	S	S	D	-	-	V	X6
Q-13P	-	-	-	S	S	E	-	-	V	
Q-12P	-	-	-	S	S	D	-	-	V	
Q-14P	-	-	-	S	S	E	-	-	V	
Q-02P	-	-	-	S	S	D	-	-	V	
Q-10P	-	-	-	S	S	E	-	-	V	
Q-15P	V	-	-	S	P	E	-	-	V	
R-01C	V	S	-	S	T	D	K	I	-	X4
R-03C	V	S	-	S	T	D	K	I	-	
R-01P	-	S	-	G	T	D	K	-	-	X5
R-13P	-	S	-	G	T	D	K	-	-	X2
R-10P	V	S	-	S	T	D	K	-	V	X2
R-05P	-	S	-	S	T	D	K	-	-	
R-09P	V	S	-	S	T	D	K	-	V	
R-12P	-	S	-	G	T	D	K	-	-	
R-03P	-	S	-	G	T	D	K	-	-	
R-11P	-	S	-	G	T	D	K	-	-	

FIG. 3—*Continued.*

	293	300	304	306	308	322	341	360	362	
HXB2	E	N	R	R	R	K	N	T	I	
JRCSF	K	S	-	S	H	E	-	-	V	
S-04C	K	H	-	S	H	D	-	-	A	X3
S-03C	K	H	-	S	H	D	-	-	A	X2
S-06C	K	H	-	S	H	D	-	-	V	
S-15C	K	H	-	S	H	D	-	-	A	
S-02C	K	H	-	S	H	D	-	-	A	
S-01C	K	H	-	S	P	D	D	-	V	
S-09C	K	R	-	S	H	D	-	-	A	
S-10C	K	H	-	S	H	D	-	-	A	
S-05C	K	H	-	S	H	D	-	-	A	
S-14C	K	H	-	S	P	D	-	-	A	
S-03P	V	-	-	S	N	D	D	-	A	X2
S-05P	V	-	-	S	N	D	D	-	A	X2
S-11P	V	-	-	S	N	D	D	-	A	
S-04P	V	-	-	S	N	D	G	-	V	
S-08P	V	-	-	S	N	D	D	-	A	
S-09P	V	-	-	S	N	N	D	-	A	
S-02P	V	-	-	S	N	D	D	-	A	
S-06P	V	-	-	S	N	D	D	-	A	
S-12P	V	-	-	S	N	D	D	-	V	

sampling CSF, the study of CSF virus provides additional theoretical advantages. Whereas parenchymal DNA can disproportionately reflect archival and in some cases replication-defective virus, RNA in CSF has been shown to turn over relatively rapidly (20, 87), reflecting the kinetics of recently formed virions, in a manner analogous to the differences observed between plasma virus and HIV DNA in peripheral blood mononuclear cells (34, 61, 88). Relatively

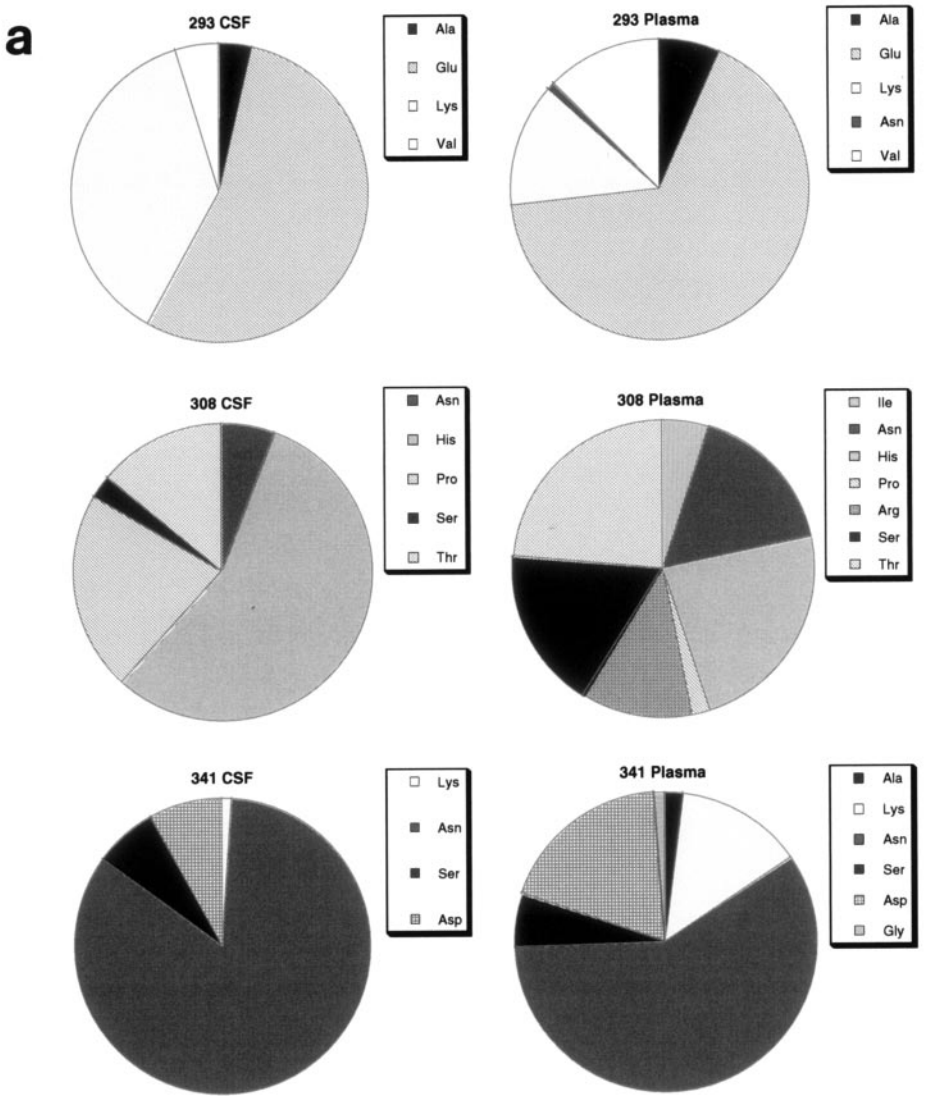

FIG. 4. AA composition at codons 293, 308, and 341. (a) Pie chart showing the percentage of CSF and plasma sequences with particular AA. Only data from subjects included in the pruned data set are included here. (b) Pie chart showing the percentage of CSF and plasma sequences carrying AA labeled according to side chain characteristics. Compositions of AA at these three sites were all significantly different for CSF and plasma virus ($P < 0.01$).

few HIV RNA genetic studies of postmortem brain specimens have been reported, possibly due to the lability of RNA and their degradation during the postmortem period. However, in the SIV-infected macaque model, DNA and RNA comprised distinct sequence variants when recovered from brain and isolated microglia of freshly euthanized animals (4). Underscoring this particular advantage of CSF RNA sequences, only 3 of the 225 CSF RNA clones we sequenced contained frameshifts or nonsense mutations compared with high frequencies of defective forms reported from prior studies of HIV DNA sequence in brain parenchyma (53, 54, 68). Thus, genetic studies of CSF HIV in subjects with relatively advanced disease complement HIV studies using brain samples from autopsies or biopsies (29, 39, 62) by providing only recently produced virions. We studied cell-free CSF samples to minimize effects from sampling of HIV sequences from lymphocytes that may traffic into the CSF. This approach may account in part for the relatively high proportion of samples demonstrating segregation of HIV populations.

Genetic studies comparing HIV from the CNS with that in blood or lymphoid organs have identified sequence differences in env, pol, gag, nef, tat, and the long terminal repeat (1, 5, 7, 13, 42, 46, 62, 66, 72, 81, 83, 99). The greatest attention has been paid to env sequences, in particular those corresponding to the V3 loop, an important (though not exclusive) determinant of both cell tropism and neutralization susceptibility by antibody (45). The reported differences could result from genetic drift of segregated populations or from selection by immune or other factors within the CNS. Our findings that C2-V3 sequences in CSF differed from those in plasma therefore corroborate many earlier studies that have catalogued genetic

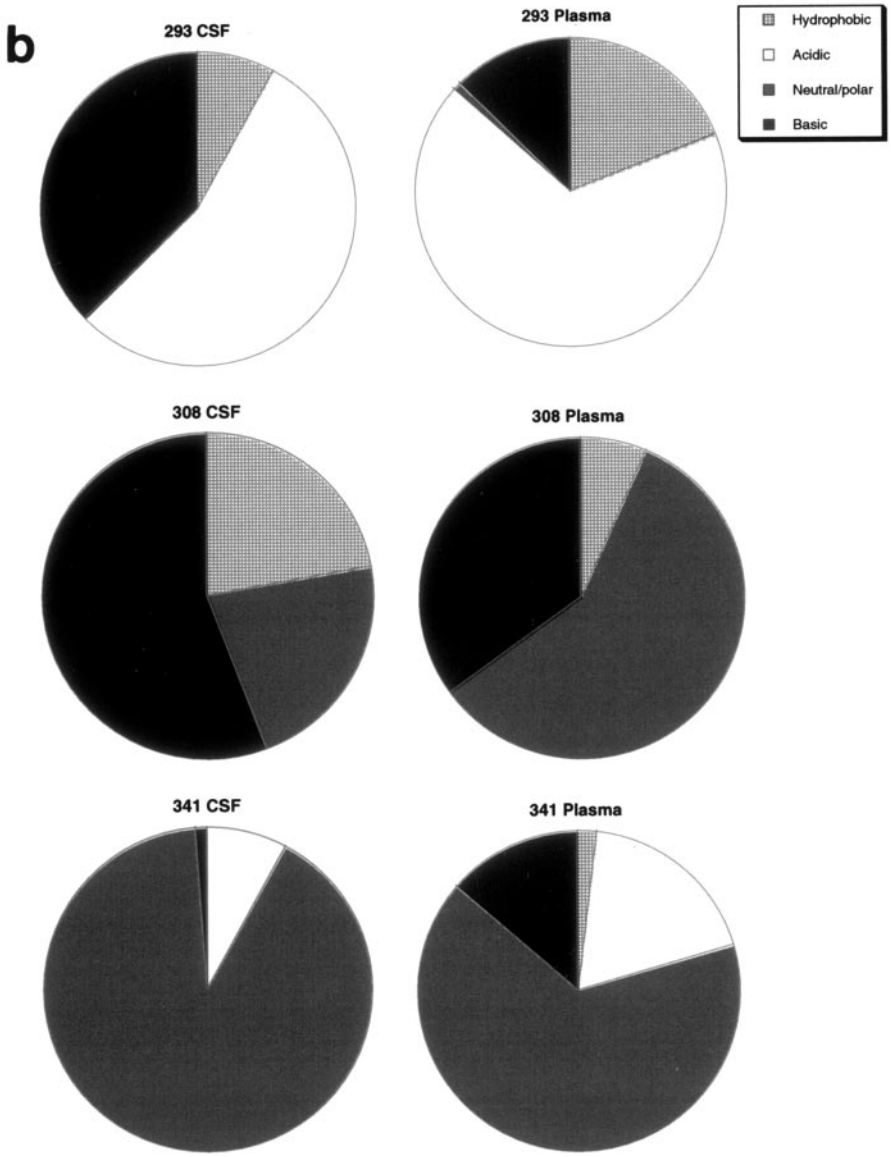

FIG. 4—*Continued.*

differences between virus in CNS and virus in blood or lymphoid tissues. However, while some earlier studies have suggested the existence of env genotypes associated with neurotropism and neurovirulence (46, 48, 73), others have disputed this (16, 66, 79). An aim of the present study was to revisit this question.

Recently proposed models for the origin of CSF virus implicate an increasing contribution of parenchymal CNS sources over peripheral lymphoid sources with advancing disease (20, 21, 87, 90). Our results are consistent with such a view, since we found a trend towards more segregation between CSF and plasma sequences in individuals with advanced disease. Dis-

TABLE 3. Summary of regression analyses categorizing AA residues by physico-chemical properties

Method	Data set	% Correct classifications	Relevant residues[a]
Stepwise logistic regression	All patients	70	293**, 308**, 341**, 360*
Regression trees	All patients	77 (13 leaves)	293, 304, 308, 341, 360
Stepwise logistic regression	Pruned	74	293**, 308**, 341**
Forward logistic regression	Pruned	81	293, 308, 341, 360, 362
Regression trees	Pruned	90 (10 leaves) or 88 (15 leaves)	293, 304, 308, 341, 360, 362

[a] **, $P < 0.01$; *, $0.05 < P < 0.01$.

TABLE 4. Resistance summary[a]

Subject	Any Rx	Current Rx	Source	NRT1 mutation(s)	NNRT1 mutation(s)	PI mutation(s)	env partitioning
A	3TC, D4T, EFV, IDV	None	Plasma	219Q	None	None	Yes
			CSF	184V, 219Q	103N	None	
B	ZDV, 3TC, ddC, ABV, DLV, EFV, NFV	ZDV, 3TC, ABV, EFV, NFV	Plasma	None	103N/K	54V, 71T/V, 82A/T, 84V, 90M	Yes¶
			CSF	None	None	None	
H	D4T, 3TC, NFV	None	Plasma	None	103N	None	Yes
			CSF	None	None	None	
M	ZDV, 3TC, NFV*	ZDV, 3TC, NFV	Plasma	None	None	77I	Yes
			CSF	None	None	None	
N	ZDV, 3TC, NFV	ZDV, 3TC	Plasma	None	None	30N, 71V, 77I, 88D	Yes
			CSF	None	None	36I	
O	None	None	Plasma	68G, 74V, 118I, 184V, 210W, 215Y	None	10I, 30N, 46I, 63P, 71T, 77I, 90M	Yes
			CSF	184V	None	10I, 71T	
Q	D4T, 3TC, NFV	None	Plasma	67N, 69D, 70R, 215F, 219Q	None	20R, 63P	Yes
			CSF	215Y	None	20R, 63P	
R	ZDV, 3TC, ddC, IDV, NFV	ZDV, NFV	Plasma	215Y	181C	63P, 77I, 90M	Yes
			CSF	None	181C	63P, 77I, 90M	
S	ZDV, 3TC, IDV	None	Plasma	184V, 215Y	None	10I, 32I, 46I, 47V, 53L, 63P, 71V, 73S, 82A/T, 84V, 90M	Yes
			CSF	None	None	63P	
D	ZDV, 3TC, ddC, NVP, SQV, NFV, IDV	None	Plasma	210W, 215Y	188L	10I, 46I, 63P, 71T, 77I, 84V, 90M	No
			CSF	210W, 215Y	188L	10I, 46I, 63P, 71T, 77I, 84V, 90M	
E	ZDV, ddI, ddC, 3TC, IDV	None	Plasma	67N, 184V, 215Y	None	10I, 77I, 82A, 90M	No
			CSF	67N, 184V, 215Y	None	10I, 77I, 82A, 90M	
L	ZDV, D4T, ddC, 3TC, ABV, EFV, NFV, RTV, SQV, APV	ABV, 3TC, APV	Plasma	67N, 118I, 184V, 210W, 215Y	103N, 108I, 181C, 190A	10I, 32I, 46I/L, 53L, 54V, 63P, 71V, 82A, 90M	Yes¶
			CSF	67N, 118I, 184V, 210W, 215Y	103N, 108I, 181C, 190A	10I, 32I, 46I/L, 53L, 54V, 63P, 71V, 82A, 90M	
K	None	None	Plasma	None	181C	10I, 82A, 90M	No
			CSF	None	181C	10I, 82A, 90M	
C	None	None	Plasma	None	None	None	No
			CSF	None	None	None	
F	ZDV, 3TC	ZDV, 3TC‡	Plasma	None	None	None	Yes
			CSF	None	None	None	
I	None	None	Plasma	None	None	None	No
			CSF	None	None	None	
J	None†	None	Plasma	None	None	None (71T)	Yes
			CSF	None	None	None (71T)	
P	D4T, 3TC, NVP, NFV	None	Plasma	None	None	None (63P, 77I)	Yes
			CSF	None	None	None (63P, 77I)	

[a] Any Rx lists antiretroviral agent use past or present; †current Rx lists antiretroviral agents or no antiretroviral agents in use for a minimum of 2 months prior to study. Numbers entered for resistance mutations correspond to AA, residues of reverse transcriptase and protease, respectively, followed by the IUPAC letter designation for the AA substitution or, when the composition at particular positions appeared to be mixed, two letters separated by a slash. Subjects J and P, who had concordant protease substitutions corresponding to frequently encountered polymorphisms (Kozal et al.) as the only resistance mutations observed, were treated as subjects without drug resistance. *, past treatment history incomplete; †, ZDV for <2 weeks in remote past; ‡, suspected Rx adherence problems due to poor memory. ZDV, zidovudine; D4T, stavudine; ABV, abacavir; 3TC, lamivudine; ddI, didanosine; ddC, zalcitabine; DLV, delavridine; NVP, nevirapine; EFV, efavirenz; IDV, indinavir; SQV, saquinavir; NFV, nelfinavir; RTV, ritonavir; APV, amprenavir. ¶, exhibited partitioning of env sequences with borderline significance, $0.5 < P < 0.01$ by Maddison-Slatkin test.

cordant resistance was encountered more frequently for individuals with a nadir CD4 of less than 200/mm^3, although this association did not reach statistical significance, owing perhaps to the small number of individuals studied with CD4 counts of >200 mm^3.

Anticipating that HIV from both systemic and CNS sources might be sampled from CSF in any given subject, we sought a CNS signature sequence in C2-V3 from both the entire data set as well as from a pruned data set by using phylogenetic criteria to exclude those individuals likely to have CSF virus produced by systemic sources. We did not find a single exclusive signature pattern that predicted the CNS origin of a virus. However, regression analyses identified a small number of positions that appeared to be important for discriminating

TABLE 5. Correlation between discordant resistance, env sequence partitioning, and neurocognitive impairment[a]

Parameter	Any Rx	Discordant resistance	V3 sequence partitioning	C2-V3 signature	Nadir CD4 >200	Current CD4 >100	Plasma RNA $>10^5$	CSF RNA $>10^4$
Discordant resistance	NS		0.035 (α)	0.007 (α)	0.09 (α^{-1})	NS	NS	NS
V3 sequence partitioning	0.09 (α)	0.035 (α)		0.008 (α)	NS	NS	NS	0.10 (α^{-1})
Neurocognitive function (GDS)	NS	0.07 (α)	NS	NS	0.05 (α^{-1})	NS	NS	NS

[a] Data are *P* values for correlation between discordant resistance and env sequence partitioning (using Fisher's exact test) and neurocognitive impairment (using Welch's analysis of variance) with clinical and virological parameters. CD4 levels are reported in cells per cubic millimeter; RNA levels are reported in log copies per milliliter. NS, not significant; α, direct correlation; α^{-1}, inverse correlation.

HIV populations from the two compartments. In related work, construction of a decision tree based on the physico-chemical characteristics of AA at six positions permitted the proper classification of up to 90% of sequences when evaluating only the pruned data (M. C. Strain, J. K. Wong and S. Pillai, unpublished data). Of note, position 308 is identical to one of two positions implicated by Power and colleagues (referred to as position 305 of the macrophage-tropic HIV consensus) to be important in determining neurovirulence (73) and to one of several positions in the V3 loop identified by Korber and colleagues as possible CNS signatures (46). Together, these findings suggest that the HIV phenotype(s) that confers neurotropism is genetically complex, and although the phenotype(s) may not be described by simple signature AA substitutions, it is nevertheless associated with discernible and predictable covariation. Further, the finding that codons 293, 308, and 341 are under positive selection and can be used to classify sequences by anatomic source based either on specific AA or physico-chemical characteristics of the AA provides compelling evidence for effects of selective forces generic to the CNS acting on these sites in the HIV envelope.

Despite the identification of residue 308 in this study as an important CNS-defining position and the implication of the same position by Powers and colleagues as a position conferring neurovirulence, we did not observe any evidence that CNS signature mutations correlated with cognitive abnormalities. Thus, we interpret these patterns as indicative of HIV neurotropism but not necessarily neurovirulence. However, such determinants of neurovirulence might be outside of the C2-V3 domain (66, 100) or might be found only in minority HIV populations that infect small but functionally critical cellular targets, such as astrocytes (60, 67, 97) and microvascular endothelial cells (89, 97). In the macaque model of neuro-AIDS, adaptation of SIV by serial animal passage in microglial cells appears to confer a phenotype that reliably produces SIV encephalitis (51, 92), providing further evidence that either or both neurotropism and neurovirulence of primate lentiviruses are selectable traits.

The seven signature positions identified in this study do not include either of the canonical positions in the V3 loop that help to define coreceptor usage (25, 85). This observation is consistent with others indicating that coreceptor usage does not discriminate HIV populations in the CNS from systemic HIV (30, 66). Phenotypic studies were not performed as a part of the present study, and such studies will be necessary to define the biologic requirements for neurotropism. Potential phenotypic characteristics for CNS adaptation might include such features as a lower requirement for primary and coreceptor density, as supported by in vitro models (19) and in vivo studies on small numbers of subjects (30). While one or more of the residues that we identified here might confer such a phenotype, they may also interact with sites outside of V3 and play indirect roles.

Segregated, CNS-adapted HIV populations in the CSF exhibited distinct resistance patterns. The high frequency of discordant resistance in our subjects, all of whom had no concurrent OIs, confirms recent results from subjects who underwent CSF sampling because of suspected and documented CNS OIs (14, 91). Concordance of resistance patterns was associated with similar V3 sequences in CSF and plasma virus, suggesting either that CNS and systemic HIV populations were not segregated or a resistant HIV variant had replaced wild-type virus in both compartments in the absence of recombination between env and pol coding regions. In contrast, we observed both discordant resistance patterns and the simultaneous partitioning of V3 sequences in the majority of cases. These cases are consistent with either the independent emergence of resistance in isolated HIV populations or the emergence of resistance in one compartment that then spreads to the other with the subsequent divergence of HIV sequences (in such cases, possibly aided by recombination). However, without longitudinal data, these possibilities cannot be distinguished.

We encountered discordant resistance both in individuals receiving failing ART at the time of sampling as well as in individuals off therapy at the time of study. This suggests that HIV populations may evolve independently during both the selection of resistance on therapy and reversion to wild-type off therapy. One subject harbored a substantially more resistant virus in CSF off therapy, while the plasma virus had apparently reverted to a less resistant sequence type. In such a scenario, treatment selection based on resistance testing of plasma virus alone would have failed to account for this highly resistant, actively replicating HIV population.

This and other recent studies of HIV in CSF reveal a high frequency of discordant resistance compared with earlier studies (84, 94). Possible explanations for these discrepant findings are differences in the complexity and composition of antiretroviral regimens the study subjects received. Whereas many earlier studies had focused on resistance to zidovudine, a drug with reasonably good CSF penetration, many contemporary regimens include drugs with marginal CSF penetration, such as the protease inhibitors (3, 28, 50, 52). Heterogeneity of drug concentrations has been predicted to promote the emergence of resistance (41) and may also explain the maintenance of populations of virus with differing resistance patterns in this and other recent studies (14, 91). Finally, the presence of discordant drug resistance was associated with more severe neurocognitive dysfunction as reflected by higher GDS. This

trend demonstrated only borderline statistical significance due to the relatively small sample size but is important to note, since subjects with more severe neurocognitive deficits for whom control of virus replication in CNS would be more clinically important appear more likely to harbor virus in the CNS that has a resistance profile discordant from that of blood virus.

In summary, our results demonstrate that CSF HIV appears genetically distinct from contemporaneous plasma HIV in a majority of individuals with advanced disease, confirming both compartmental segregation and autonomous replication within the CNS. Furthermore, a small number of signature positions in and near the V3 loop may reflect genetic adaptation to replication in the CNS. An important correlate of independent V3 genotypes was the frequent observation of discordant resistance patterns between virus in plasma and CSF. These findings may have important implications for drug treatment and subject monitoring. For example, drug failure based on plasma HIV resistance pattern might not mean that CSF HIV has also developed resistance. Continuation of drugs that were previously effective may be indicated for CNS protection, even as new agents are added to address drug-resistant plasma virus. Clearly, establishment of the clinical significance of such discordant resistance patterns will need to await results of detailed longitudinal studies. Nevertheless, these results help to substantiate the value of CSF sampling in studies aimed at elucidating HIV neuropathogenesis and guiding neuro-AIDS treatment.

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