HIV dementia patients exhibit reduced viral neutralization and increased envelope sequence diversity in blood and brain

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**Objectives:** To examine the relationship between the humoral immune response and viral envelope diversity among HIV/AIDS patients with or without HIV-associated dementia (HAD).

**Methods:** Whole blood and sera were collected from age- and disease-progression matched AIDS-defined patients with and without neuro-cognitive impairment at two centers. Peripheral blood mononuclear cells were isolated from whole blood and separated into monocyte/macrophage and peripheral blood lymphocyte (PBL) preparations. Genomic DNA, isolated from the PBL population, was used as template to amplify HIV-1 C2V3 envelope sequences in a nested PCR protocol. The resulting fragments were sequenced and subjected to a phylogenetic analysis.

**Results:** Sera from non-demented (ND; \(n = 21\)) patients neutralized infection of CCR5-dependent, but not CXCR4-dependent viruses, more efficiently than sera from HAD patients (\(n = 15\)) \((P < 0.05)\). A recombinant virus containing a brain derived C2V3 sequence was also neutralized less efficiently by sera from HAD patients \((P < 0.05)\). C2V3 envelope sequences amplified from PBL revealed significantly greater diversity within the V3 region from HAD compared with ND patients \((P < 0.001)\). The number of non-synonymous substitutions was positively correlated with the severity of neuro-cognitive impairment of patients \((P < 0.005)\). Similarly, brain derived V3 sequences exhibited significantly increased diversity among HAD patients \((P < 0.001)\).

**Conclusion:** Our findings imply that HAD patients exhibited impaired serological responses that may lead to the emergence of viral mutants that potentially could infect the brain and mediate neurodegeneration.

**Keywords:** HIV sequence variability, envelope proteins, neutralization, neurological/brain, HIV-associated dementia, chemokine receptor

**Introduction**

Disease progression during HIV-1 infection is accompanied by an increasing diversity in viral sequences found within the infected host [1]. The error-prone reverse transcriptase-mediated step in the HIV-1 life cycle is responsible for the high mutation rate [2]. HIV-1 sequences within patients slowly progressing towards AIDS become more diverse over time [3–8] compared with those within rapidly progressing patients, partly due to the extended time of immunocompetence and amplified immune responses [8,9]. Among HIV/AIDS patients who receive antiretroviral therapy, viral sequences will also evolve over time in...
genes not targeted by the drugs and despite suppression of the viral load to undetectable levels [10–12]. The immune response, the availability of target cells for infection and viral fitness determine which viral variants arise during the course of infection [13–16]. Differences in selection pressure within HIV-1-infected patients may influence disease progression, because of the potential for generation of more pathogenic virus variants [16–18].

Earlier studies have addressed the relationship of HIV diversity and progression towards AIDS within the host [3–6,9], but little is known about the relationship of viral diversity across hosts and the onset of HIV-induced neurological disease. Infection by HIV-1 causes inflammation within the brain and neuronal degeneration [19], resulting in HIV-associated dementia (HAD) or the less severe minor cognitive and motor disorder (MCMD) [20–22]. Molecular diversity in both viral and host genes has been implicated in the pathogenesis of HAD [23–29]. Infection of the brain by HIV-1 and subsequent production of viral proteins, including Env and Tat, are known to initiate a cascade of cell signalling events that lead to the production of molecules with putative neurotoxic actions, culminating in neuronal degeneration or death [19,30,31].

Given that the host immune system is an important factor controlling HIV diversity and pathogenesis, differences in humoral and cellular immune responses could select for or against viruses that infect the brain and cause neurological damage. As the viruses present in the brain are generated in the blood and then cross the blood–brain barrier by an unresolved mechanism [32], differences in neutralization by antibodies in the serum may influence sequence variability of the HIV-1 envelope in blood and determine which viruses infect the brain. Therefore, we examined differences in viral neutralization by sera from patients with and without HAD and its relationship to virus sequence diversity. The C2V3 region of the HIV-1 envelope was found to be associated with differences in neutralization between groups. In addition, the C2V3 sequence of the HIV-1 envelope amplified from blood exhibited differences in groups. In addition, the C2V3 region of the HIV-1 envelope was found to be associated with differences in neutralization between groups. Therefore, we examined differences in viral neutralization by sera from patients with and without HAD and its relationship to virus sequence diversity. The C2V3 region of the HIV-1 envelope was found to be associated with differences in neutralization between groups. In addition, the C2V3 sequence of the HIV-1 envelope amplified from blood exhibited differences in groups.

Materials and methods

Patients

Informed consent was obtained from 48 HIV-1 sero-positive AIDS-defined patients from the St Michael’s Hospital HIV Neuropsychology Laboratory (Toronto, Ontario, Canada) and the Southern Alberta Clinic (Calgary, Alberta, Canada) for participation in this study, and studies were performed with approval from the Ethics committees of both institutions. Two centers were used to avoid any bias arising from geographical or demographic factors. Patients were diagnosed as ND (n = 23), MCMD (n = 8) and HAD (n = 17), using established criteria, as described previously [20,22]. In addition, neurological impairment was assessed by standard neuropsychological testing [33,34]. Care was taken to ensure that patients in the different groups did not differ in their rate of systemic disease progression and patients with clinically significant neuropathy were excluded. All groups were matched for age, CD4 cell count, viral load (Table 1) and none had opportunistic infections of the brain, which were excluded in all patients by neuroimaging. All patients had received highly active antiretroviral therapy (HAART) or were still receiving HAART at the time of blood collection. HAART consisted of at least two nucleoside analogues, usually zidovudine, stavudine or lamivudine, and a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor.

The brain derived sequences used for comparative analysis were derived from a different cohort of patients consisting of eight HAD and seven ND patients described previously [25] and selected using the same criteria as described above.

Neutralization assays

Sera collected from HAD and ND patients were heat inactivated at 56°C. Serial dilutions (minimum 1 : 8, maximum 1 : 128) of the sera were prepared in culture.
media and mixed with virus containing media using fixed amounts of virus (1 x 10^3 focus forming units/ml). The virus-serum mixture was incubated for 2 h at 37°C and then used to infect HeLa CD4/CXCR4/C CR5 cells [35] for 5 h at 37°C in a 5%/95% CO2/air atmosphere. Sera from HIV-1 seronegative individuals were used as negative controls. Two days after infection cells were fixed in 95% ethanol and immunostained using a p24/25 antibody (AIDS Research and Reference Reagent Program, NIAID, NIH, catalogue #384/#4250) and the number of p24/25 positive foci were counted. Neutralization was represented as percent foci reduction relative to number of foci formed by the virus in the absence of patient sera. All neutralization assays were performed in quadruplicate. The viruses used in the neutralization assays were NL4-3 [36], YU-2 [37], and NL-J1 (a recombinant NL4-3 containing the C2V3 envelope region of JR-FL [38]).

Isolation and preparation of peripheral blood lymphocytes and monocytes/macrophages

Whole blood was collected and peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma, St. Louis, Missouri, USA). Subsequently, cells of monocyte/macrophage lineage were separated from peripheral blood lymphocytes (PBL) by attachment or using a CD14-specific antibody conjugated to paramagnetic beads using the manufacturers protocols (Dynal Inc., Lake Success, New York, USA). This procedure resulted in two cell preparations one consisting of PBL depleted for > 99% of monocytes/macrophages (M/MΦ) and another consisting of cells of primarily monocyte/macrophage lineage (95–99% pure). For each patient group a number of replicate samples were collected and processed.

PCR and sequencing

Genomic DNA from the PBL and M/MΦ preparations was isolated using DNAzol (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s protocols. The HIV-1 C2V3 envelope region from the integrated proviral DNA was amplified using a previously described nested PCR protocol [25], which consisted of 35 cycles for first- and second-round PCR for the PBL and 40 cycles each for the M/MΦ. The annealing temperatures for the first- and second-round PCR were 45 and 50°C, respectively. PCR fragments were isolated from gel, the incomplete fragment ends were filled in with Klenow, phosphorylated using T4 polynucleotide kinase, and cloned into the EcoRV site of pBluescript SKII(+). All reagents were obtained from New England BioLabs Ltd. (Mississauga, Ontario, Canada) and used to the manufacturer’s specifications. PCR fragments were sequenced directly in both directions using the PCR primers or multiple clones of the cloned PCR fragments were sequenced using M13 reverse and forward primers (Invitrogen). DNA sequences were determined by automated sequencing on an ABI 370 sequencer (Applied Biosystems, Foster City, CA, USA) using the manufacturers protocols and reagents. The HIV-1 C2V3 sequences from replicate samples at sequential dates (8.6 ± 1.7 months) were obtained and analysed for 10 patients (four ND, three MCMD and three HAD). These serial samples were similar in nucleotide sequence (d = 0.0116 ± 0.0037) and clustered together in a bootstrap analysis of phylogeny (bootstrap values > 95, data not shown) with the sequences obtained after the first blood draw, confirming the validity of the amplified sequences. As an additional control, the cloned C2V3 region of HIV NL4-3 was subjected to the same nested PCR protocol as the patient DNA samples and subsequently sequenced (data not shown). This analysis was repeated several times and did not reveal any differences in the sequence of the obtained PCR products from that of the original sequence, which would exclude mutations introduced by the PCR as an explanation for any observed difference in sequences. The sequences obtained from cloned and PCR fragments were aligned using DNASTar version 3.82 (DNASTar Inc., Madison, Wisconsin, USA) and the consensus sequence obtained for each patient was used for phylogenetic analyses. All sequences have been submitted to Genbank (accession numbers AF503213–AF503276).

Sequence and phylogenetic analysis

The brain derived C2V3 envelope sequences and the consensus sequences obtained for the C2V3 region from blood were analysed using the MEGA version 1.01 and MEGA version 2.0 software packages [39,40]. Mean total (d), non-synonymous (dN, i.e., codon changing substitutions) and synonymous (dS, i.e., non-codon changing substitutions) distances for the C2 and V3 regions [41] were calculated for each patient group by pair-wise comparison and pair-wise gap stripping of each sequence with the other sequences within the same group. Similarly, the mean dS/dN ratio within each group (ND, MCMD and HAD) was also calculated. Any bias towards non-synonymous or synonymous substitutions, results in a different dS/dN ratio that gives information about the selections pressures acting on a given replicating sequence [16]. The MEGA software was also used to construct neighbour-joining trees using the Jukes–Cantor correction with 1000 replicates for the bootstrap analysis.

Statistical analysis

All statistical analyses were performed using Graphpad InStat Version 3.01. (GraphPad Software, San Diego, California, USA) and P < 0.05 were considered significant.
Results

Neutralization of HIV by sera of ND and HAD patients

Previous studies suggest that the humoral immune response influences HIV-1 related disease occurrence and progression [8,42–46]. To explore this concept further and its relationship to HIV neuropathogenesis, differences in neutralization ability of the sera from HAD (n = 15) and ND (n = 21) patients (Table 1) were compared for two HIV-1 virus strains, NL4-3 and YU-2 (Fig. 1). We focused on the HAD and ND patients, as they represent well defined endpoints of the spectrum of HIV-1 associated neurological disease. NL4-3 [36] is a prototype HIV-1 strain that uses CXCR4 as co-receptor for viral entry and was neutralized equally efficiently by the sera of HAD and ND patients at all dilutions (Fig. 1). Sera of both groups neutralized HIV-1 more efficiently than that of HIV-1 seronegative patients (data not shown). In contrast, the brain derived strain from a patient with HAD, YU-2 [37], which uses CCR5 as co-receptor, was consistently neutralized significantly less efficiently by sera of HAD patients at all dilutions ($P < 0.05$ and $P < 0.001$). A similar effect was observed for neutralization studies using HIV-1 JR-FL (data not shown), which is also a brain derived CCR5-dependent strain from a patient with HAD [47]. Because the V3 envelope region is an important neutralizing determinant for HIV-1 in vitro [48,49], the extent to which this difference was dependent on the V3 region was assessed using a recombinant virus, HIV NL-J1, in neutralization assays. NL-J1 is a recombinant virus in which the C2V3 region of NL4-3 has been replaced by the C2V3 region of the envelope of HIV-1-JR-FL and it is CCR5-dependent [38]. NL-J1 also appeared to be neutralized significantly less efficiently at dilutions 1:8, 1:16 and 1:32 by the sera of HAD patients ($P < 0.05$). As NL4-3 and the recombinant virus NL-J1 differ only in their C2V3 envelope sequences and not in their Gag and Pol sequences, they are equally sensitive to antiretroviral drugs present in patients' sera. Hence the observed focus reduction was probably the consequence of differences in neutralizing molecules present in the patient serum rather than the presence of antiretroviral drugs. Analyses of the dilution at which there was a 50% reduction in foci formation revealed that YU-2 required significantly lower dilutions for HAD derived sera compared with ND derived sera ($P < 0.05$), confirming the above findings. These results indicated differences in the neutralizing ability between sera of HAD patients and ND patients, which was strain-dependent and that the C2V3 envelope region was a determinant in discriminating the serological responses between patients with or without HAD.

![Graph](image_url)

Fig. 1. Neutralization, represented as percentage foci reduction, of HIV-1 strains NL4-3, YU-2 and NL-J1 by sera from ND (n = 21) and HAD (n = 15) patients at different sera dilutions. HIV-1 YU-2 was consistently neutralized less efficiently by different dilutions of sera from HAD patients than by the sera from ND patients. NL-J1 was neutralized less efficiently by sera of HAD patients than by sera from ND patients at serum dilutions of 1:8, 1:16 and 1:32. No difference was observed in neutralization of NL4-3 by the sera of HAD and ND patients. *$P < 0.05$, **$P < 0.001$, Mann–Whitney U test.
Phylogenetic analysis of proviral DNA sequences derived from PBL

As differences in virus neutralization were associated with the C2V3 envelope region, the extent of viral molecular diversity in this region was examined by comparing the C2V3 proviral sequences found in the PBL of ND, MCMD and HAD patients (Table 1). The MCMD patients were included as a group of AIDS-defined patients with intermediate severity of HIV-induced neurological disease. For all 48 HIV/AIDS patients the C2V3 envelope region could be amplified from PBL using the present nested PCR protocol. As amplification of the C2V3 region of M/Mφ was highly variable per patient, resulting in limited number of M/ Mφ sequences, and contamination with PBL could not be excluded, these sequences were not used for further phylogenetic analysis.

Common sequences or amino acid residues were not observed within the different groups, nor was there a difference in overall charge, hydrophobicity or potential glycosylation of the V3 region among the different patient groups (Fig. 2). However, a higher number of amino acid residue substitutions were observed in V3 sequences of the HAD patients, in which 15 out of the 17 patients had more than three amino acid residue substitutions relative to the overall consensus sequence versus nine out of 23 for the ND patients (P < 0.01; Fisher's exact test). No significant difference was observed for the MCMD patients compared to either of the other groups. The number of unique amino acid residues, found in two or more patients in one group and not in the other groups was higher, although not significant, among the HAD patients (seven unique residues) compared to ND patients (three unique residues). Analysis of coreceptor use mediated by the amplified sequences performed by inserting the C2V3 region encoding PCR fragments from four patients of each group into a HIV-1 molecular clone derived from NL4-3 [24], revealed use of CCR5 primarily as co-receptor for viral entry (data not shown). No relationship between co-receptor use and neurological status was observed in this subset of patients, similar to previous studies of brain derived C2V3 sequences [24, 50, 51].

A neighbour-joining tree for the consensus nucleotide sequences of the V3 region obtained from PBL (L) and M/ Mφ (M) of 48 patients by group, together with the consensus sequence. Sequences marked 2 were obtained from subsequent samples. Relative to the overall consensus sequence, more amino acid substitutions were present in the sequences obtained from HAD patients than in those from the ND patients (P < 0.01, Fisher's exact test). The unique amino acid residues in one group but not present in other groups at the same positions are indicated by circles.

Using the MEGA software the mean total (d), non-synonymous (dN) and synonymous (dS) pair-wise dis-

Fig. 2. Alignment of the inferred consensus amino acid sequences of the V3 region obtained from the PBL (L) and M/ Mφ (M) of 48 patients by group, together with the consensus sequence. Sequences marked 2 were obtained from subsequent samples. Relative to the overall consensus sequence, more amino acid substitutions were present in the sequences obtained from HAD patients than in those from the ND patients (P < 0.01, Fisher's exact test). The unique amino acid residues in one group but not present in other groups at the same positions are indicated by circles.

Distances [41] for the C2 and V3 region consensus sequences obtained from the ND, MCMD and HAD patients were calculated (Table 2). The mean d and the dN were significantly higher (P < 0.001) for the V3
Fig. 3. Phylogenetic neighbour-joining tree (using Jukes–Cantor correction) based on the consensus envelope V3 region sequences obtained from PBL of the 48 patients enrolled in this study. Patient numbers and diagnosis are indicated. Also included are the V3 regions of HIV-1 D clade virus strain NDK [69]. The tree is rooted against the V3 envelope sequence of the HIV-1 D clade virus strain YU-2 and JR-FL. The tree is rooted against the V3 envelope region of the HIV-1 D clade virus strain NDK [69]. Although no specific pattern of clustering within patient groups was observed by bootstrap analysis using 1000 replicates for both the C2 and V3 regions, the branches corresponding to HAD and MCMD patients were relatively more extended than those of the ND patients.

As the mean \(d_N\) values for the HIV-1 envelope V3 region of MCMD and HAD patients compared with those of the ND patients. The mean \(d_s\) for the V3 region was significantly higher for the MCMD patients compared to ND and HAD patients (MCMD versus HAD, \(P < 0.01\); MCMD versus ND, \(P < 0.001\)). No significant difference was observed between the \(d_s\) of the V3 region of the HAD and ND cases. For the C2 region, the mean \(d_s\) and \(d_N\) values were significantly higher for the ND cases. This was most pronounced for the mean \(d_s\) of the C2 region (ND versus HAD, \(P < 0.01\); ND versus MCMD and HAD versus MCMD, \(P < 0.001\)), for which the \(d_s\) was highest for the ND and lowest for the MCMD patients. The mean \(d_s\) for the C2 region of the HAD patients was significantly higher (\(P < 0.001\)) than that of the MCMD patients but significantly lower than the mean \(d_s\) for the ND patients (\(P < 0.01\)).

An indicator of bias towards amino acid changing mutations resulting from differing selection pressure is the ratio between the \(d_N\) and \(d_s\) [16]. Table 2 shows that the mean \(d_N/d_s\) for the V3 region in HAD patients was significantly higher than the MCMD and ND patients (\(P < 0.001\)). Although the mean \(d_N/d_s\) value for the C2 region was significantly higher (\(P < 0.001\)) for the MCMD patients than for the HAD and ND patients, it was \(< 1\), and thus is indicative of negative or purifying selection [16]. The mean \(d_N/d_s\) values for the C2 region were not significantly different for the MCMD and ND patients. In addition, no significant differences in transversion and transition rates for the C2 and V3 region were observed among the three groups (data not shown).

### Table 2. Mean distance (± SEM) among C3 and V3 sequences within groups. \(P\) values were calculated using Dunn’s multiple comparison test.

<table>
<thead>
<tr>
<th>Envelope region</th>
<th>Blood</th>
<th>Brain [25]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND (n = 23)</td>
<td>MCMC (n = 8)</td>
</tr>
<tr>
<td>C2</td>
<td>0.0919 ± 0.0008</td>
<td>0.0819 ± 0.0027</td>
</tr>
<tr>
<td></td>
<td>0.1625 ± 0.0002</td>
<td>0.1212 ± 0.0053</td>
</tr>
<tr>
<td></td>
<td>0.0697 ± 0.0008</td>
<td>0.0664 ± 0.0025</td>
</tr>
<tr>
<td></td>
<td>0.4620 ± 0.0078</td>
<td>0.6136 ± 0.0362</td>
</tr>
<tr>
<td>V3</td>
<td>0.0985 ± 0.0018</td>
<td>0.1489 ± 0.0115</td>
</tr>
<tr>
<td></td>
<td>0.1106 ± 0.0031</td>
<td>0.1759 ± 0.0123</td>
</tr>
<tr>
<td></td>
<td>0.0959 ± 0.0019</td>
<td>0.1401 ± 0.0112</td>
</tr>
<tr>
<td></td>
<td>1.0232 ± 0.1341</td>
<td>0.8666 ± 0.0496</td>
</tr>
</tbody>
</table>

\(\text{a}\)For \(d_C\) blood: \(P < 0.01\) non-demented (ND) versus minor cognitive and motor disorder (MCMD), \(p < 0.001\) ND versus HIV-associated dementia (HAD). \(\text{b}\)For \(d_C\) C2 blood: \(P < 0.01\) ND versus HAD, \(P < 0.001\) ND versus MCMD and MCMC versus HAD. Brain: \(P < 0.001\) ND versus HAD. \(\text{c}\)For \(d_C\) blood: \(P < 0.01\) ND versus HAD. \(\text{d}\)For \(d_s\) and \(d_N\) C2 blood: \(P < 0.001\) MCMC versus ND/HAD. Brain: \(P < 0.01\) ND versus HAD. \(\text{e}\)For \(d_s\) B V3 blood: \(P < 0.001\) ND versus MCMC/HAD. Brain: \(P < 0.001\) ND versus HAD. \(\text{f}\)For \(d_s\) and \(d_N\) V3 blood: \(P < 0.001\) ND/MCMC and Brain: \(P < 0.01\) ND versus HAD.
region was significantly higher for both the MCMD and HAD patients, with values > 0.1, the possibility of a correlation between the mean dN for the V3 region obtained from a patient and the severity of neurological disease was explored. Although not the basis for the diagnosis of HAD, a reliable measure of neurocognitive impairment is the mean deficit score (MDS), obtained by combining the results of three neuropsychological tests for both motor and cognitive function of the patient [34,34]. The mean dN for the V3 region of all the individual patients was plotted against their MDS score data, revealing a correlation (Spearman’s r = 0.434; P < 0.005) between the dN for the V3 region and neurological disease severity. In contrast, no such correlation was found between the time of AIDS onset, age, duration of HAART, and time since seroconversion and dN values. Interestingly, two of the ND patients with a mean dN value > 0.1 subsequently have progressed to HAD. These patients, 344 and 001, had dN values of 0.1668 ± 0.0061 and 0.1509 ± 0.0087, respectively, indicating that the V3 dN value may be a predictor of brain disease progression. In summary, the higher dN and the dN/dS value > 1 for the V3 envelope region suggested that this domain was subject to greater selection pressure [16] in HAD patients than in the ND and MCMD patients.

Comparison of proviral sequences derived from brains of ND and HAD patients

To determine if the above phenomena were also apparent in brain derived HIV-1 sequences, C2V3 envelope sequences amplified from the brain of a different cohort of ND and HAD patients [25] were analysed (Table 2; ND clone numbers (n = 7): 10-1, 17-2, 49-1, 1-4, 14-1, 36-1, 48-1; HAD clone numbers (n = 8): 12-1, 2-1, 26-1, 20-1, 19-1, 34-1, 9-1, 15-1). These latter sequences had not been subjected to phylogenetic analysis in the past. The mean d of the V3 region for the HAD patients was significantly higher (P < 0.001) than for the ND patients. For the C2 region the mean d was significantly higher (P < 0.01) for the ND patients. In addition, for the V3 sequences obtained from the HAD patients, the mean dN was significantly higher (P < 0.001), while the mean dS was significantly higher (P < 0.001) for the C2 region for the HAD patients. No significant differences were observed for the dN/dS of the C2 region and d of the V3 region between the ND and HAD patients.

By calculating the mean dN/dS (Table 2) for each group, it was observed that the mean dN/dS value for the V3 region among the HAD patients was significantly higher (P < 0.001) than for the ND patients. The differing mean dN/dS value suggested that the V3 sequences in the brain were subject to a different selection pressure in the HAD patients. Although the mean dN/dS was significantly larger for the C2 region in the ND patients (P < 0.001) it was < 1, suggesting that negative selection was acting on this region for both clinical groups. This analysis indicated that the C2V3 envelope sequences found in the brain varied more among the HAD patients compared to the ND patients, with a tendency towards larger diversity and more non-synonymous substitutions in the V3 region, similar to observations for the blood derived sequences.

Discussion

In the current study, we showed that HAD patients exhibited impaired serological responses against CCR5-dependent virus strains and increased viral diversity was observed in HAD derived V3 sequences, which may reflect the emergence of viral mutants. Many studies have established a link between lower efficiency in virus neutralization and HIV-1 non-neurological disease progression [8,43–46]. Our results indicated that sera from well-defined ND and HAD patients neutralized a non-brain derived CXCR4-dependent HIV-1 strain (NL4-3) with similar efficiency, but a brain derived CCR5-dependent HIV-1 strain (YU-2) was neutralized less efficiently by the sera of HAD patients. These observations complement a previous report [52] demonstrating that sera from HIV-1 seropositive patients with neurological disease exhibited a reduction in viral neutralization compared to patients without neurological disease, in serum dilution ranges similar to our observations. Although the differences in neutralization of laboratory strains observed between HAD and ND patients in this study and by Beilke et al. [52] may be modest, they are significant, and imply that HAD patients are less able to produce sufficient or specific antibodies with a neutralizing action against viruses destined to infect the brain. As the HAD and ND groups in the current study did not differ in rate of systemic disease progression (Table 1), the serological response differences may have contributed to their neurological status. Differences in humoral and cellular immune responses to, and neutralization sensitivity of, CCR5 and CXCR4 using HIV-1 strains have been postulated as selection pressures favouring CCR5-dependent viruses [13,53]. In the context of our findings, this may be more pronounced in HAD patients enabling CCR5-dependent viruses to escape and enter the brain, perhaps also due to increased immune tolerance to CCR5-dependent viruses, as they have been present since the onset of infection in latent reservoirs in the body [54].

The reduced virus neutralization with recombinant virus NL-J1 for the sera from HAD patients, indicated a role for the C2V3 envelope region and could be directly associated with the increased viral diversity in the PBL derived sequences observed for the HAD and
MCMC patient groups. The viruses found in the brain are primarily macrophage-tropic and use CCR5 as coreceptor for entry [24,55,56]. In the PBL cell population CCR5 is expressed on resting memory T-cells, and during different stages of T-cell development [54,57,58]. As these PBL act as viral reservoir [54], the viruses infecting these cells are likely to be important for infection of the brain and development of HAD; this is also supported by studies of HIV-related neuropathology in which lymphocytes were detected in the brain parenchyma [59,60].

Several studies have reported a link between increasing HIV diversity and progression towards AIDS [3–6,9], although it has been suggested that differences in viral diversity could be an epiphenomenon of increasing immunodeficiency [1]. However, no differences in rates of systemic disease progression were observed between groups in this study. Hence, our findings of differences between groups of patients rather than within a patient, suggest that the increased blood derived viral variability in the HAD/MCMD patients might be directly associated with neuropathogenesis. The \( \frac{d_N}{d_S} \) values observed for the V3 sequences implied that the V3 region in the HAD patients was subject to different selection pressures than the V3 regions in the MCMC/ND patients. The \( \frac{d_N}{d_S} \) value for the C2 envelope region indicates a negative or purifying selection acting on this envelope region in all patient groups. As the V3 region is an important determinant for viral neurotoxicity [24,61], the difference in selection pressure within patient groups has important implications for the neuropathogenesis of HIV-1. Increased viral sequence diversity in the V3 region may be relevant, because it increases the likelihood of generating viral strains that may infect the brain more efficiently or are more capable of causing neuronal death [24,62,63].

The relationship observed between increased viral molecular diversity and HAD is complemented by the correlation between the \( d_N \) value for the V3 region for each patient and their matched MDS score. None of the other variables (i.e. viral load, CD4 cell count, age, HAART duration) were correlated with the \( d_N \) for the V3 region. Moreover, the observation that two ND patients (344 and 001), with high \( d_N \) values (> 0.1) for the V3 region, subsequently progressed towards HAD, strengthens the possible link between viral variability in the V3 region and HAD. The potential utility of the \( d_N \) value of the V3 region in predicting occurrence and progression of HAD is currently under investigation. An additional corroboration is the similar pattern in viral diversity observed for the brain derived sequences from HAD and ND patients from a different cohort [25], especially as it has been demonstrated that these sequences differ in their ability to induce neuronal death [24]. The differences were less pronounced for the sequences derived from the brain, reflecting less selection pressure acting on HIV-1 within the brain, similar to previous findings of spleen and brain derived HIV-1 envelope sequences of different clades [38].

Our data also suggest that selection by the immune system is not the only factor influencing viral diversity. A higher level of replication of HIV-1, which leads to increased viral diversity [16], may explain the high C2 region \( d_N/d_S \) ratio and the high \( d_N \) and \( d_S \) for the V3 region in the MCMC patients. Although no detectable viremia may be present, active replication can occur elsewhere in the body among patients receiving HAART [64,65]. This would explain why patients already on HAART may develop HAD [66]. The present finding of increased molecular variability in the HIV-1 C2V3 region in MCMC and HAD patients may be the result of viral replication in different compartments in the body combined with different selection pressures from the immune system. In this respect, antiretroviral therapies using structured therapeutic interruptions [67,68] and enhanced viral resistance to antiretroviral drugs could actually increase viral diversity. This will be important for the current and future therapies for HIV-1 infection, as increasing viral sequence diversity is associated with a greater risk of developing HIV-related neurological disease.

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