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Short communication

PCR detection of host and HIV-1 sequences from archival brain tissue

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Mutations in CCR5 and CCR2b have been recently shown to affect disease progression towards AIDS. A role for these host genotypes in AIDS dementia complex (ADC) has also been postulated but remains unclear. Additionally, brain-derived envelope sequences from HIV-1 have been associated with ADC but their specific contribution to pathogenesis remains uncertain. This study demonstrates the successful use of PCR techniques to isolate host CCR5 and CCR2b, and HIV-1 V3 sequences from paraffin embedded tissues from patients with and without ADC. PCR amplification from archival tissue offers a novel approach for studying the interactions between potential neuroprotective elements in the host and virulence determinants in HIV that may contribute to differences in susceptibility to ADC. *Journal of NeuroVirology* (2000) **6,** 164–171.

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HIV-1 frequently infects the central nervous system, causing a spectrum of clinical, pathological, and metabolic abnormalities, including dementia (AIDS Dementia Complex, ADC) (Navia *et al*, 1986a,b; Price, 1995). For reasons that remain poorly understood, at least 30% of infected individuals with advanced AIDS will never suffer clinical or pathological evidence of neurological damage. By comparison, nearly 5% of infected patients will develop dementia as their AIDS defining illness. The cause of these differences in susceptibility to ADC and brain pathology remains unknown.

Initial infection by HIV as well as the progression towards AIDS has been attributed to specific chemokine receptor genotypes based on the recent discovery of mutations in genes coding for these receptors (Samson *et al*, 1996; Dean *et al*, 1996; Huang *et al*, 1996; Smith *et al*, 1997; Rizzardi *et al*, 1998; Kostrikis *et al*, 1998). A 32 base pair deletion in CCR5, termed CCR5-Δ32, has been shown to render homozygotes immune to HIV infection (Samson *et al*, 1996; Dean *et al*, 1996; Huang *et al*, 1996). Further, the frequency of the heterozygous form, present in 10–20% of the general population, was found to be significantly higher in infected individuals with a median survival time of 10 years or more, suggesting that CCR5-Δ32 may act in a dominant fashion to delay progression to AIDS (Samson *et al*, 1996). A mutation in CCR2b, termed CCR2-64I, resulting from a conservative substitution of a valine at position 64 to an isoleucine, has also been shown to delay the median time of progression to AIDS in both heterozygotes and homozygotes (Smith *et al*, 1997; Rizzardi *et al*, 1998; Kostrikis *et al*, 1998) whereas a polymorphism in the promoter region of the CCR5 gene, termed CCR5P1, has been associated with rapid progression to AIDS (Martin *et al*, 1998).

Several studies have identified chemokine receptors in brain, including CXCR4, CXCR2, CCR5, and CCR3 (Lavi *et al*, 1997, 1998; Taouik *et al*, 1998; Vallat *et al*, 1998; He *et al*, 1997; Sanders *et al*, 1998). CCR5 has been identified as the predominant co-receptor for HIV entry into microglia, although other co-receptors such as CCR3 may also be involved (Albright *et al*, 1999; Sheih *et al*, 1998; Ghorpade *et al*, 1998; He *et al*, 1997). One hypothesis to explain the differences in suscept-

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ibility to ADC is that host chemokine receptor genotypes may correlate with the degree to which persons with AIDS will develop brain injury and cognitive impairment.

Two groups have recently examined the relationship between CCR5 genotype and the risk for ADC, based on the hypothesis that CCR5-Δ32 heterozygosity may protect against AIDS dementia. Barroga *et al* (1997) reported no difference in CCR5 genotype in 45 patients with HIV associated neurocogntiive disorders compared to the general population. In contrast, van Rij *et al* (1998) reported a trend for lower frequencies of CCR5-Δ32 in ADC subjects (4%) compared to non ADC patients (15%). Additional studies in this area may help to resolve these conflicting results.

Additionally, viral factors, particularly sequences within the HIV envelope gene (*env*) may contribute to HIV neuropathogenesis (LaRosa *et al*, 1990). Early studies of the V3 loop showed evidence that brain-derived isolates evolve independently as a quasispecies when compared to spleen derived sequences (Li *et al*, 1991; Epstein *et al*, 1991). More recent analysis has shown regional compartmentalization of HIV sequences in brains of patients with ADC (Chang *et al*, 1998; Shapshak *et al*, 1999). Further, conserved amino acid signatures in the envelope region of brain isolates have been associated with ADC (Gartner *et al*, 1997; Korber *et al*, 1994; Power *et al*, 1994, 1998; Shapshak *et al*, 1999). Specifically, Power *et al* (1994) identified a significantly greater frequency of histidine at position 305 and leucine at position 329 in subjects with dementia when compared to subjects without dementia. Studies performed by others, however, have failed to detect such associations (Reddy *et al*, 1996; Di Stefano *et al*, 1996). Thus the existence of neurovirulent sequences in the HIV genome remains controversial and will require further analyses of well-characterized subjects combined with *in vitro* studies of such putative sequences (Power *et al*, 1998; Ohagen *et al*, 1999).

Together these studies suggest that both viral and host factors may contribute to differences in susceptibility to ADC and brain injury. Further studies of this important tissue, however, have been limited by the general lack of available frozen brain tissues. To circumvent this problem, we have adapted a potentially useful PCR-based approach to isolate host and viral sequences from archival tissues derived from HIV-infected patients who have been characterized in terms of relative levels of brain pathology and neurological impairment.

Paraffin embedded archival tissue from seven HIV positive patients and three noninfected normal controls, were obtained for CCR5 and CCR2b genotyping. Of the seven HIV-infected subjects, five showed no evidence of cognitive impairment and their brains were histologically normal while the remaining two had severe ADC and HIV encephalitis. In addition, DNA from basal ganglia, frontal cortex, visual cortex, and spleen from one of the severely demented patients was extracted for V3 sequence isolation. The post mortem interval (PMI) ranged from 16–48 h, with the exception of one sample with a 6 day PMI. All samples were fixed in 10% buffered formalin from 3 days to 3 weeks.

DNA was obtained from paraffin-embedded tissues according to the methods described by Wright and Manos (1990), with minor modifications. Briefly, six 10 μM sections were obtained from paraffin embedded brain or spleen tissue and pooled. This approach optimized DNA yield, and combined with the phenol-chloroform extractions described below, helped increase the quality of the DNA sample. Sections were placed into autoclaved microcentrifuge tubes using autoclaved tooth picks, which had not been in contact with previous samples. Sections were extracted twice for 30 min with 1.5 ml of xylene to remove the paraffin. Samples were washed twice with 1 ml, 100% ethanol and subsequently dried in a vacuum drier. Samples were digested overnight at 37°C with 300 μl of 200 μg/ml Protinase K diluted in digestion Buffer (50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween 20, 1% SDS). Following digestion, samples were incubated at 95°C for 8 min and spun to collect undigested tissue. Supernatants containing DNA were further purified by phenol: chloroform: isoamyl alcohol extraction. The organic phase was back extracted with TE, and the aqueous phases were combined. This solution was extracted once more with phenol-chloroform, followed by extraction with chloroform. The DNA was concentrated using a standard ethanol precipitation method and resuspended in 50 μl of dH2O.

CCR5Δ32 genotype screening was performed according to Liu *et al* (1996). Primer sequences were as follows: SP4.760 5′-CTTCATTACACCTGCAGCTCT-3′ and PM6.942 5′-CACAGCCCTGTGCCTCTTCTTC-3′. Five to 10 μl of DNA from paraffin embedded tissues was amplified in a reaction mixture containing 1 × PCR buffer (Gibco); 1 μM of each primer; 200 μM of each dATP, dCTP, dGTP, dTTP; 2 mM $MgCl_2$; and 2 U of Taq Polymerase (Gibco). A positive control reaction was performed with 100 nanograms of DNA extracted from peripheral blood lymphocytes using the Puregene kit (Gentra Systems). This sample had previously been identified as a heterozygote while testing the SP4.760 and PM6.942 primer pair. A negative control was also performed where no DNA was included in the reaction mixture. Samples were denatured for 2 min at 94°C, the Taq polymerase was added, then the following reaction was carried out for 32 cycles: 94°C, 1 min; 58°C, 30 s; and 72°C for 30 s. The reaction was completed with a 4 min extension reaction at 72°C. PCR Products were resolved on a 2% agarose gel and stained with ethidium bromide.

Polymorphism screening for CCR2-64I was performed as described by Smith *et al* (1997). The following primers were used to amplify a 128 bp portion of the CCR2b gene: CKR2_1A=5′-TTGTGGGCAACATGaTGG-3′ and CKR2_1Z=5′-GAGCCCACAATGGGAGAGTA-3′. The CKR2_1A primer contains a mismatched adenine which introduces a *BsaB*I restriction site if the CCR2b gene codes for an isoleucine at position 64 of the protein instead of a valine. The following cycling conditions were used to amplify this fragment in a 50 μl reaction containing 1 × PCR buffer (Gibco); 3 mM $MgCl_2$; 200 μM dATP, dTTP, dGTP, and dCTP; 1 μM of each primer; and 2 U Taq polymerase (Gibco): 94°C for 2 min followed by 40 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 30 s, and a final extension of 4 min at 72°C. The $MgCl_2$ concentration was optimized for amplification of DNA extracted from paraffin. Twenty μl of the PCR product was digested with 20 U of *BsaB*I overnight at 60°C in a 30 ul reaction volume. This yielded 110 bp and 18 bp products in the polymorphic variant. The undigested and digested fragments were resolved on a 4% agarose gel and stained with ethidium bromide and compared to a control sample known to be heterozygous for this mutation (identified when testing the reaction conditions on DNA isolated from peripheral blood lymphocytes).

V3 sequence isolation was performed according to the procedure described by Power *et al* (1994). Rogers *et al* (1990) reported less efficient PCR amplification of viral sequences using DNA isolated from samples fixed in formalin for periods longer than 1 week. In this study, viral DNA was successfully isolated from samples from a patient with a 3 week fixation time. Other studies have also shown that the length of fixation does not seem to impair the ability to amplify host or viral sequences from paraffin embedded brain tissue (Nicoll *et al*, 1991, An *et al*, 1994). Nested PCR amplification was used to isolate the C2 and V3 regions of the HIV-1 *env* gene. The following primer pair was used to perform the first PCR reaction: V3-ext-1: 5′-GTAA AA TTAACCCCAC TCT GTGT-3′; V3-ext-2: 5′-CGTG CGGCCCTC AACATTT AAAACTGTGC-3′. A 100 ul reaction containing 1 × PCR buffer (Gibco); 1.5 mM $MgCl_2$; 1 μM of each external primer, 1.5 U Taq Polymerase (Gibco), and 5 μl of the DNA isolation as a template. Five μl of the PCR product from the first reaction was used as a template to amplify the C2 and V3 regions using the following interior primer pair: V3-int-1 5′-TTTGCTAGC-TATCTGTTTTAAAGT-3′; V3-int-2 5′-TTACA-CAGGCCTGTCCAAAGG-3′. This PCR fragment was isolated from a 1% agarose gel using the QiaexII Gel extraction Kit (Qiagen) and cloned into the pCR 2.1 plasmid using the InVitrogen TA Cloning kit. Two clones from each of the brain regions were double strand sequenced (ABI50 automated sequencer). Sequences were subject to BLAST searches in order to determine similarity to other viral isolates, and further analyzed using DNA Strider 1.2 to determine the V3 amino acid sequences.

To perform phylogenetic analysis, sequences were aligned using ClustalX (Thompson *et al*, 1997) (Figure 3). The alignment contained no ambiguities. Because these sequences were closely related, the method of Templeton *et al* (1992) instead of neighbor-joining or parsimony techniques was used to reconstruct evolutionary relationships. This method has been shown to have greater statistical power than other methods when sequences have few differences among them (Crandall 1994, 1995, 1996; Crandall *et al*., 1994). Using this approach, connections were made between sequences by minimal mutational steps. These connections were supported at a greater than 95% confidence level (Templeton *et al*, 1992) and were made unambiguously. This method was implemented in PAUP* 4.0b (Swofford, 1998). Nonsynonymous (nucleotide substitutions resulting in an amino acid replacement) and synonymous (nucleotide substitutions not resulting in an amino acid replacement) substitutions were calculated by mapping the changes on the phylogeny and counted directly (Crandall *et al*., 1999a).

Host chemokine receptor genotypes were successfully amplified from paraffin embedded tissues of all subjects studied. The length of fixation or PMI was not a factor in the ability to amplify host sequences from these samples. CCR5-Δ32 mutation screening results revealed that this mutation was absent in the HIV positive and normal controls, as compared to a control sample that was heterozygous for this allele. For CCR2b mutation screening, all patients tested were homozygous for the normal allele as compared to a heterozygous positive control (Figure 1).

V3 sequences were also isolated from the basal ganglia, frontal cortex, visual cortex, and spleen of one patient. Most nucleotide substitutions in the V3 sequences resulted in amino acid substitutions in the C2 sequence adjacent to the V3 region, with the exception of one clone from the visual cortex which showed a substitution of an arginine in place of glycine at position 320. Brain derived sequences displayed significant similarity to JR-CSF, an M-tropic viral isolate from the CSF of a patient with severe ADC. These brain-derived sequences shared the reported conserved histidine at position 305, but not the proposed conserved leucine at position 329 (Power *et al*, 1994). The spleen derived sequence was also similar to the JR-CSF V3 sequence (Figure 2).

Phylogenetic analysis showed that sequences isolated from frontal cortex, basal ganglia and visual cortex were all closely related to each other (only a single mutational step differentiating different sequences) as well as to the spleen isolated

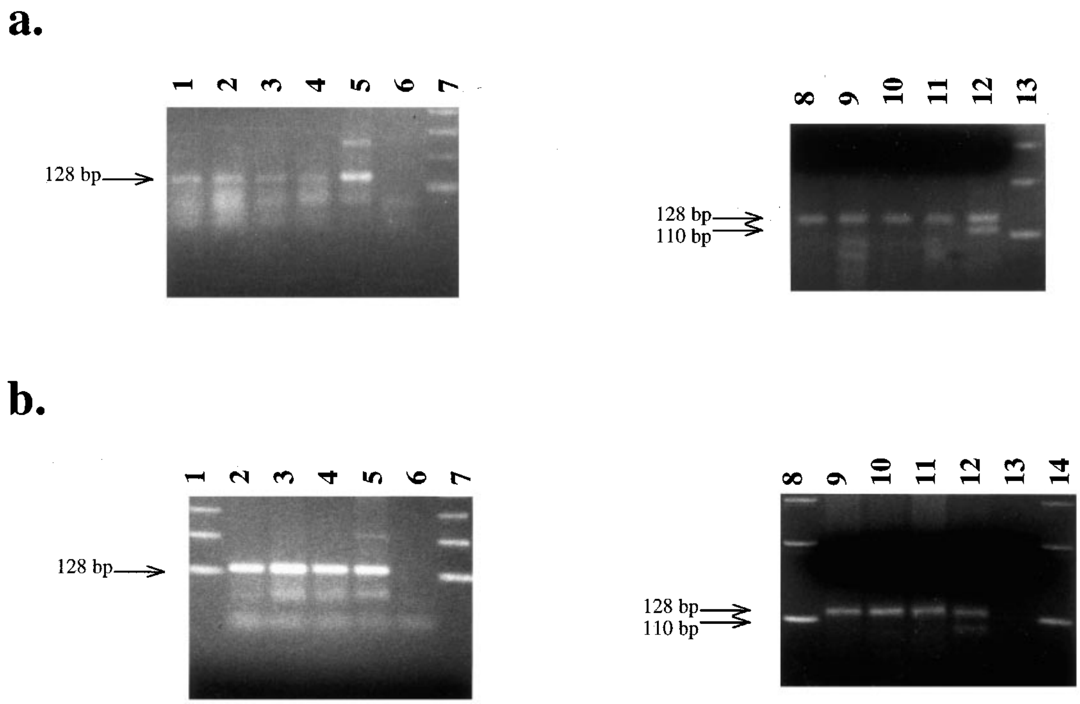

Figure 1 CCR2-64I polymorphism screening. (**a**) CCR2-64I polymorphism screening of four HIV positive subjects using DNA from paraffin embedded tissue. Lanes 1–6: PCR products prior to restriction digest. Lanes 8–12: PCR products from lanes 1–5 following restriction digest with *BsaB*I. Lanes 7 and 13: 123 bp DNA ladder (Gibco BRL). Lane 6: PCR product from the negative control (no DNA template added). Lanes 5 and 12: PCR product from the positive control, a known heterozygote, before and after restriction digest. The HIV positive subjects in lanes 1–3 and 8–9 did not have dementia. The subject in lanes 4 and 11 had severe ADC. (**b**) CCR2-64I polymorphism screening of three normal control subjects using DNA from paraffin embedded tissue. Lanes 2–6: PCR products prior to restriction digest. Lanes 9–13: PCR products following restriction digest with *BasB*I. Lanes 1, 7, 8, and 14: 123 bp DNA ladder (Gibco BRL). Lanes 2–4 and 9–11: PCR products from normal control subjects before and after restriction digest. Lane 6 and 13: PCR product from the negative control before and after restriction digest. Lanes 5 and 12: PCR product from the positive control before and after restriction digest.

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                                                                 [         305                    ] 329
Mø   :CTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSENFTNNAKTIIVQLNESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNNTLKQIAS
FC-2 :-K----------------------------K-----N---D-------------K------S-------H---------------------------Q----------
FC-3 :-K----------------------------K-----D---D-------------K------S-------H---------------------------Q----------
VC-1 :-K----------------------------K-----D---DD------------K------S-------H---------------R-----------Q----------
VC-3 :-K----------------------------K-----D---D-------------K------S-------H---------------------------Q----------
BG-1 :-K----------------------------K-----D---D-------------K------S-------H---------------------------Q----------
BG-3 :-K----------------------------K-----D---DD------------K------S-------H---------------------------Q----------
SP-4 :-K----------------------------K-----N---D-------------K------S-------H---------------------------Q----------
JRCF :-K----------------------------K-----D---D-------------K------S-------H---------------------------Q---------E
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Figure 2 V3 amino acid sequence comparison from frontal cortex (FC), basal ganglia (BG), visual cortex (VC), and spleen (SP) of a patient with severe dementia. Mø is an archetypal M tropic strain. JR-CF is a viral isolate from the CSF of a patient with severe dementia (JR-CSF). The V3 loop sequence is within brackets. The proposed conserved histidine at position 305 is highlighted in bold. Position 329 is the proposed site of a conserved leucine.

sequence (Figure 3). The majority of these nucleotide substitutions were nonsynonymous, which indicated strong directional selection acting on these sequences (Messier and Stewart 1997; Sharp 1997). These results suggest ample gene flow among brain regions, contrary to other reports (Chang *et al*, 1998; Shapshak *et al*, 1999). Although such conclusions are limited due to the small sample size (n=2) from each brain region. It is interesting to note that no two sequences from the same tissue region cluster together before clustering with another tissue type. Indeed, a frontal cortex isolated sequence was identical to a spleen-isolated sequence and the other frontal cortex sequence was identical to a visual cortex sequence. It is noteworthy that this subject had developed progressive dementia as the only AIDS defining illness until death. Additional studies of such patients and

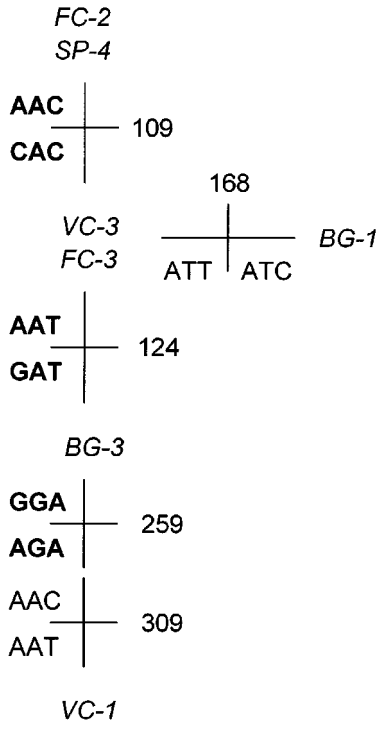

Figure 3 Genealogy of the V3 sequences isolated from frontal cortex (FC), basal ganglia (BG), visual cortex (VC), and spleen (SP). The phylogenetic relationships in this unrooted network were estimated using the method of Templeton *et al* (1992). Support for all connections was at 95% confidence or higher. There is no inferred directionality. The mutational differences between sequences are shown along the branches with the codon closest to a given haplotype reflecting its sequence. Nucleotide sequences are to the left or bottom of the branches. The codon positions are to the right or above the branches. For example, the sequence of FC-2 has AAC at codon 109 whereas VC-3 has CAC. Those nucleotide substitutions in bold resulted in an amino acid change (nonsynonymous substitutions).

sampling may allow further discrimination of these viral populations.

This preliminary study is the first to report the successful isolation of chemokine receptor and V3 sequences from paraffin embedded tissues of HIV-infected patients. The postmortem interval or length of fixation did not affect the ability to detect these sequences, consistent with prior reports (Nicoll *et al*, 1991; An *et al*, 1994). One previous study by An *et al* (1994) reported the successful amplification of the HIV-1 *gag* gene from fresh frozen and paraffin embedded brain tissues of HIV positive patients. The use of PCR to amplify and isolate viral sequences from paraffin embedded tissue has been applied in a number of other instances (Taubenberger *et al*, 1997; Vago *et al*, 1996; Vesy *et al*, 1993). Notably, influenza sequences were detected from paraffin embedded tissue of a patient who died of the 1918 'Spanish' influenza in an effort to identify molecular determinants of this highly virulent strain. Sequence analysis allowed classification of the infecting stain as a novel H1N1 influenza A virus belonging to a subgroup that infects both humans and swine (Taubenberger *et al*, 1997).

Although the sample size was too small to draw any significant conclusion, the ability to perform CCR5 and CCR2b mutation screening on DNA extracted from archival tissue in the present study represents a novel approach to study the genetic association of these host factors to ADC. This strategy could also be used to study other potential host factors such as SDF-1 or CCR5 promoter variants that have been shown to affect the course of HIV infection (Winkler *et al*, 1998; Martin *et al*, 1998).

The relative contributions of host genotypes to susceptibility to HIV-1 infection and progression is an emerging issue (Crandall *et al*, 1999b). The results presented here demonstrate the feasibility of using PCR techniques to detect both host and HIV-1 sequences from archival paraffin embedded tissue. This strategy offers several advantages: the number of well characterized cases with fixed tissues is far greater than what is available with frozen tissues; this would significantly expand the source of accessible brain tissues that cover a range of clinical and pathological disease. Thus the potential role of these genotypes can be addressed in a manner that takes into account differences in clinical state and brain pathology between patients – an issue that has remained poorly understood since the initial description of the disorder (Navia *et al*, 1986b). Furthermore, the use of paraffin embedded tissue would make it feasible to perform genotype analyses in a population that has been clinically and pathologically characterized prior to the advent of antiretroviral therapies. Such a strategy would help avoid the confounding effects of treatment on assessing the clinical and pathological stage (Navia *et al*, 1986b; Brew *et al*, 1995). Finally, the capacity to isolate envelope sequences from peripheral and brain tissues will permit further *in vitro* studies of their pathogenic contribution to neurological injury (Power *et al*, 1998; Ohagen *et al*, 1999).

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