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Short Communication

Human immunodeficiency virus type 1 DNA and RNA load in brains of demented and nondemented patients with acquired immunodeficiency syndrome

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The relationship between dementia and human immunodeficiency virus type 1 (HIV-1) cerebral load is not clearly understood. We used immunohistochemistry and competitive polymerase chain reaction to evaluate the density of gp 41 immunostained cells and the amount of HIV-1 DNA and RNA in the midfrontal gyrus of 21 HIV-1 infected patients, nine of whom were demented. The amounts of HIV-1 DNA and RNA, and the density of gp 41-positive cells were significantly linked. In this small series of cases, (1) although as a mean, there was a larger viral load in demented patients than in nondemented, this did not reach the significance level (2) discrepancies appeared in the population under study, some demented patients having low viral loads.

Keywords: neuroAIDS; viral burden; dementia; competitive PCR

The late stages of human immunodeficiency virus (HIV) infection are variously associated with neurological dysfunction including dementia, vacuolar myelopathy and sensory neuropathy. HIV-associated dementia ('HIV-associated cognitive and motor complex' or 'acquired immunodeficiency syndrome (AIDS)-dementia complex'; Navia *et al*, 1986; Working group of the American Academy of Neurology AIDS task force, 1991) is estimated to affect 15–30% of HIV-infected patients from Europe and North America in the late stages of AIDS (McArthur *et al*, 1993). Clinically, this syndrome is characterized by various degrees of impaired memory and concentration, psychomotor slowing, ataxia, tremor and dementia (Navia *et al*, 1986; McArthur, 1987; Working group of the American Academy of Neurology AIDS task force, 1991).

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Pathological changes possibly due to HIV-1 infection of the central nervous system (CNS) can be found in 70–90% of HIV-1-infected individuals at post-mortem examination (Budka *et al*, 1991; Spencer and Price, 1992). The mechanisms leading to dementia are, as yet, poorly understood. HIV-1 probably invades the CNS soon after infection (Spencer and Price, 1992). Neuropathological examination of AIDS patients shows that cerebral lesions are frequently found in subcortical areas (deep gray matter or white matter). Neuronal loss in the cortex has been reported (Masliah *et al*, 1992; Everall *et al*, 1994), although this has been questioned (Seilhean *et al*, 1993). Multinucleated giant cells, which are specific of HIV encephalitis, as well as diffuse myelin pallor, occur in only 50% of demented patients (Glass *et al*, 1993). Microglial cells, macrophages and, rarely, vascular endothelial cells and astrocytes can be infected (Vazeux *et al*, 1987; Saito *et al*, 1994; Tornatore *et al*, 1994). Neurons are thought to be spared.

The relationship between brain viral load, HIV-1-induced neuropathological changes and dementia are not clearly understood. Recent studies have shown that increased plasma viral burden, particularly RNA load, correlates with clinical progression in HIV-1-infected individuals (Connor

et al, 1993; Piatak *et al*, 1993; Mellors *et al*, 1996). Measurement of plasma HIV-1 RNA is more sensitive than quantitation of either HIV-1 p24 core antigen in plasma or infectious virus in blood. HIV-1 amount in brain might also correlate with the degree of CNS dysfunction. Although HIV-1 can be detected in the CNS of most AIDS patients, dementia failed to correlate with HIV DNA load and with gp41 positive cell number in the brain (Achim *et al*, 1994; Glass *et al*, 1995; Johnson *et al*, 1996).

To our knowledge, quantitation of HIV RNA amount has not yet been investigated in the brains of demented and nondemented patients with AIDS. Therefore, in order to approach the role of brain viral burden in HIV-associated dementia complex, we chose to measure, by quantitative polymerase chain reaction (PCR) analysis, both HIV-1 proviral DNA and RNA amounts in the brains of 21 demented and nondemented HIV-1 positive patients.

A cohort of 21 patients without brain focal lesion was selected for this study from 457 HIV-positive individuals, examined in R Escourolle Neuropathology Department at La Salpêtrière Hospital from 1984 to February 1996. Postmortem delay was below 48 h. Twenty had died with AIDS, and one was asymptomatic HIV-1 positive at the time of death. The population was divided in two groups: demented, nondemented – according to clinical examination following the DSM-IV criteria (American Psychiatric Association, 1994) and neuropsychological tests. Mini mental state (MMS) test has been performed in 16 cases (Table 1). All individuals with MMS scores above 24 (n=9) were found nondemented (Anthony *et al*, 1982). Two cases, already included in a previous study, underwent a large battery of psychometric tests other than the MMS and were considered as moderately and severely demented, respectively (Seilhean *et al*, 1993). Cognitive decline was neither related to age, risk factor, nor immunosuppression level.

For this study, a sample from the middle frontal gyrus was collected in a standardized fashion, frozen and stored at -80°C until nucleic acid extraction. Samples from the midfrontal, superior temporal and cingulate gyri, hippocampus, occipital cortex, nucleus basalis of Meynert, head of the caudate nucleus, pallidum, white matter of the centrum ovale, dentate nucleus, spinal cord at cervical, thoracic and lumbar levels, had been fixed in formaldehyde, included in paraffin, cut at 7 μM thickness before staining. The neuropathologic diagnoses were based on the criteria of a consensus

Table 1 Clinical and pathological data. All HIV-1 positive patients died with AIDS excepted case 21 who was asymptomatic at the time of death. Cases 3 and 4 were from subsaharian origin

Cases	*Sex*	*Age*	*Risk factor*	*CD4*	*Psychometric evaluation*	*Neuropathologic diagnosis*	*Associated findings in other areas*	*HIV DNA (log)*	*HIV DNA (log)*	*gp 41*
1	M	32	D	4	0	HIV encephalitis	–	1	5.5	12.5
2	M	67	H	64	13	HIV encephalitis	–	3.5	4.2	3.2
3	F	26	?	202	18	Diffuse poliodystrophy	Cryptococcosis	0.4	0.5	0.66
4	M	34	H	3	19	Diffuse poliodystrophy	CMV ventriculitis	0.2	0.7	1.1
5	M	49	D	0	20	Diffuse poliodystrophy	–	2.6	1.7	6
6	M	26	D	0	22	Diffuse poliodystrophy	Toxoplasmosis scar	1.9	3	5.4
7	M	26	D	50	23	Diffuse poliodystrophy	–	1.7	2.3	2.5
8	M	50	?	50	25	Diffuse poliodystrophy	CMV ventriculitis	0.6	3.4	0
9	M	47	H	48	26	Diffuse poliodystrophy	Toxoplasmosis scar	0.8	0.6	3.1
10	M	37	H	3	28	Diffuse poliodystrophy	CMV ventriculitis+ Cryptococcosis	2	4.2	2.8
11	M	41	H	10	28	Diffuse poliodystrophy	–	0	0	0.5
12	M	38	H	119	29	Diffuse poliodystrophy	Central pontine myelinolysis	0	1.3	0.9
13	M	40	B	9	29	Diffuse poliodystrophy	–	0.3	3.4	0
14	F	37	T	29	29	HIV encephalitis	–	2.1	0.3	2
15	M	47	H	4	30	Diffuse poliodystrophy	CMV ventriculitis	0.2	0	1.2
16	M	52	H	0	30	Diffuse poliodystrophy	–	0.6	0	1.6
17	M	47	H	64	***	HIV leucoencephalopathy	–	0	0.3	3.1
18	M	45	?	3	*	HIV leucoencephalopathy	CMV ventriculitis	0	1.4	0.5
19	M	32	H	22	ND	Diffuse poliodystrophy	–	0	0	0.42
20	M	28	H	180	ND	Normal		0	0	0.16
21	M	60	H	335	ND	Normal		0.7	0	0.25

Sex: M=male; F=female. *Age*: in years. *Risk factor*: B=bisexual; D=drug addict; H=homosexual; T=infected by transfusion; ?=unknown. *CD4*: CD4 lymphocyte count in blood per mm^3. *Psychometric evaluation*. Last score obtained with Mini Mental State (MMS) examination (score/30). Patients 17 and 18 underwent a battery of psychometric tests other than MMS and were found moderately (*) and severely (***) demented (Seilhean *et al*, 1993). ND=not determined. *Neuropathologic diagnosis*. According to consensus criteria (Budka *et al*, 1991). *HIV DNA*: decimal logarithm of the number (+1) of DNA copies of HIV per μg of extracted DNA. *HIV RNA*:decimal logarithm of the number (+1) of RNA copies of HIV per μg of extracted RNA. *gp 41*: number of gp 41-positive cells per mm^2

report (Budka *et al*, 1991) and are shown in Table 1. Five patients (1, 2, 14, 17, 18) had characteristic features of HIV-encephalitis including multinucleated giant cells and migroglial nodules, associated with marked myelin pallor in patients 17 and 18. Fourteen others had cortical and subcortical astrogliosis associated with cortical spongiosis, referred to as diffuse poliodystrophy.

One hundred mg of midfrontal gyrus were homogenized in lysis buffer (10 mM Tris-HCl pH 8, 100 mM EDTA pH8, 0.5% SDS, 20 μg/ml bovine pancreas RNase-DNase free (Boeringher®), 200 μg/ml proteinase K (Boeringher®). DNA was extracted as previously described (Maniatis *et al*, 1982). All PCR reactions were performed with 0.5 μg DNA. Quality of extracted DNA was assessed by amplification of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene using the human G3PDH amplimer set of Clontech® (USA). Reactions were always carried out in a total volume of 50 μl containing 0.5 μg DNA sample following the manufacturer's instructions. Samples were amplified by using a DNA Thermal cycler 2400 (Perkin Elmer Cetus®, Norwalk, CT). PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

Quantitative competitive PCR amplification was performed using Clontech® (USA) PCR MIMIC technique (Siebert and Larrick, 1993; Piatak *et al*, 1993). The set of primers used to amplify a 114 bp HIV gag sequence was SK38/SK39 (Genset®, France). Competitive PCR was performed using SK38/SK39 to amplify both the target HIV gag cDNA and another internal standard constructed DNA fragment, the amount of which was known. The competitive internal standard, PCR MIMIC, was realized following the manufacturer's instructions. Briefly, it was obtained by amplification of a non homologous DNA fragment (BamHI/EcoRI fragment of v-*erb*B) using the two composite primers, exhibiting the target DNA primer sequence (SK38 and SK39) each one followed by 20 nucleotides that hybridize to opposite strands of v-*erb*B DNA fragment (antisense SK38 MIMIC: 5′ATAATCCAACCTATCCCAGTAGGAGAAATCG-CAAGTGAAATCTCCTCCG-3′; sense SK39 MIMIC:5′TTTGGTC CTTGTCT TATGTCCAGAATGCT TTCATCTCCCTGTATAACA-3′). PCR MIMIC is 256 bp and was produced in R Escourolle Laboratory so as to be easily distinguishable from the target fragment on an agarose gel. Reactions were carried out in a total volume of 50 μl containing 0.5 μg DNA sample and 2 μl of 9 serial tenfold dilutions of PCR MIMIC (1X: 1 attomole/μl) following Clontech® instructions. Forty amplification cycles were used with the following conditions: 35 s at 94°C, 1 min 45 s at 56°C, 55 s at 72°C, with an initial denaturation time of 3 min 20 s and a final extension step of 10 min. HIV DNA levels were then determined by gel analysis of the PCR products derived from the MIMIC and target.

Specificity of PCR amplification was checked by Southern blot hybridization of the PCR products with a digoxygenin (DIG)-labeled SK 39 probe (Genset®, France), using Amersham® detection kit.

One hundred mg of midfrontal gyrus were homogenized in guanidium isothiocyanate. Total cellular RNA was isolated by phenol-chloroform extraction and then precipited with isopropanol as previously described (Chomczynski and Sacchi, 1987). The reverse transcription of 1 μg of total RNA to cDNA was performed as described (Lazarini *et al*, 1996) in a total volume of 50 μl. Two μl of cDNA solution were used in each 50 μl PCR reaction, containing 2 μl of MIMIC dilutions. Nine serial tenfold dilutions of MIMIC (1X=1 attomole/μl) were carried out for each cDNA sample, and PCR was performed and analyzed as described for HIV DNA quantitation.

HIV immunoreactivity was sought in paraffin sections of the midfrontal gyrus from 21 AIDS cases using a monoclonal antibody directed against the gp41 antigen (Dupont®: 1 : 100). Immunohistochemistry for CMV early and late antigens was performed with a monoclonal antibody (Dako®, 1/600) on paraffin sections. A biotinyled anti-mouse IgG (Amersham®, 1 : 200), detected with streptavidin-peroxidase (Amersham®, 1 : 400), was used as secondary antibody. Counting of HIV positive cells was performed at X100 magnification (X10 objective) within fields of 0.735 mm^2. The mean (number/mm^2) and standard error of the mean (s.e.m.) (21 cases) were calculated for 12 fields/case.

Non parametric tests were used for statistical analysis. Groups were compared with the Mann-Whitney U test and interrelationships of quantitative variables were analyzed with the Kendall rank correlation coefficient, computed with Statview 4.02 software. Values of $p \leqslant 0.05$ were considered significant.

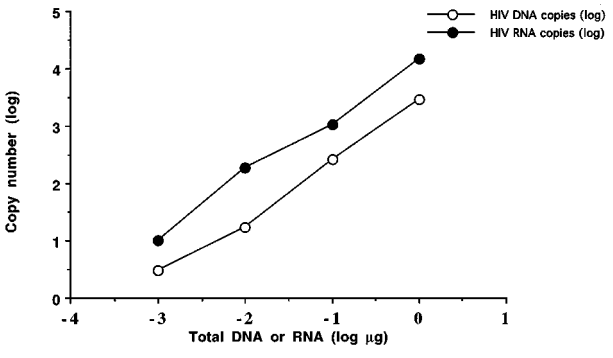

Figure 1 Competitive PCR quantitation of HIV DNA and RNA. Dilutions (1, 0.1, 0.01, 0.001 μg) of DNA and RNA extracted from frontal cortex of case 2 were performed before reverse transcription and competitive PCR. HIV DNA and RNA levels were determined by gel analysis of the PCR products.

Figure 1 shows the linearity of the competitive PCR assay in both increasing DNA and RNA levels. HIV-1 sequences (DNA or RNA) were detected in midfrontal gyrus in 18 of 21 HIV-1 infected cases, including the asymptomatic HIV-1 positive individual, in variable levels. A significant correlation, shown in Figure 2, was found between HIV DNA and RNA amounts (n=21, τ=0.441, p=0.0051). A significant correlation was also found between the density of gp41 positive cells and either DNA (n=21, τ=0.424, p=0.0063) and RNA HIV load (n=21, τ=0.305, p=0.042) in the midfrontal gyrus.

No correlation was found between dementia, assessed by MMS, and HIV nucleic acid amounts in brain (Figure 3). Both HIV RNA and DNA levels were increased, but not significantly, in the brains of demented individuals (MMS $\leqslant$ 24), compared to nondemented ones (Figure 3). These levels were found to be relatively low (HIV DNA $<$ 10 copies/μg DNA and HIV RNA $<$ 50 copies/μg RNA) in 4 individuals (cases 3, 4, 17 and 18, described in Table 1) among the nine cases with AIDS severe dementia. Conversely, high levels in viral burden (HIV DNA $>$ 100 copies/μg DNA) were also observed in the brains of two nondemented cases (MMS $>$ 24; cases 10 and 14). No correlation was found between dementia and the density of gp41 positive cells in the midfrontal gyrus. No correlation was found between the density of CD4 lymphocyte in the blood, and viral load.

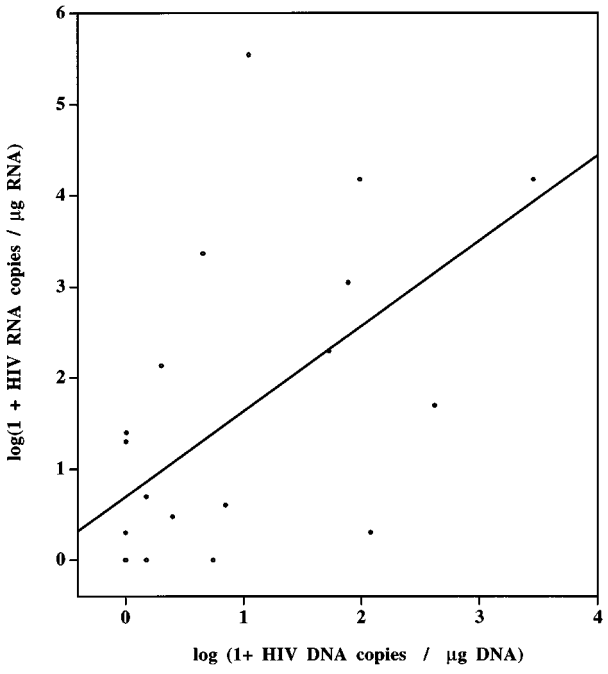

Figure 2 Correlation between HIV DNA and RNA load in the frontal cortex of 21 HIV-1 infected patients. Data are expressed as decimal logarithms of the number (+1) of either DNA copies of HIV per μg of extracted DNA or RNA copies of HIV per μg of extracted RNA. n=21; τ=0.441; p=0.0051.

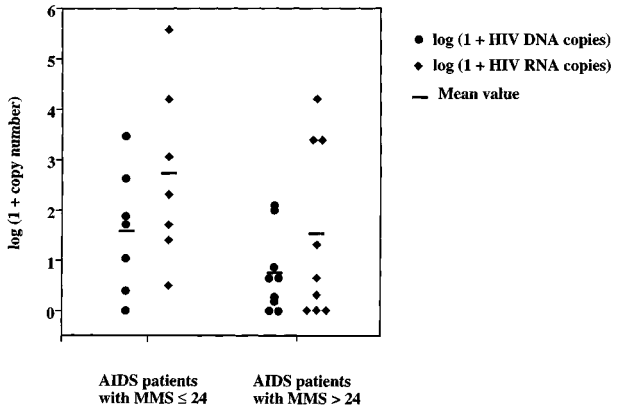

Figure 3 HIV DNA and RNA quantitation in frontal cortex of 16 AIDS patients. The AIDS cases were subcategorized: nine AIDS cases without dementia (MMS $>$ 24) and seven AIDS cases with dementia (MMS $\leqslant$ 24). Results are decimal logarithms of mean values (+1) of either DNA copies of HIV per μg of extracted DNA or RNA copies of HIV per μg of extracted RNA. The power of the test is 0.67 for DNA and 1.11 for RNA respectively.

To our knowledge, this is the first quantitation of HIV-1 RNA copies in the brains of demented and nondemented patients. A good correlation was observed between the RNA, DNA and the gp41 data, whereas no correlation was found between the presence of dementia and HIV load in the brain, assessed by quantitation of either DNA, RNA and gp41 positive cells in midfrontal gyrus. The small number of cases does not permit rejection of the hypothesis that HIV dementia is related to viral load in some cases. Nevertheless, previous observations showed a lack of correlation between HIV DNA load, gp41 positive cell number, and dementia (Glass *et al*, 1995; Johnson *et al*, 1996). Our RNA data reinforce the hypothesis that HIV dementia is not the direct consequence of virus replication in the brain.

The mechanisms leading to AIDS-associated dementia remain as yet unknown. HIV-1 may have a direct or indirect role in CNS dysfunction related to dementia. The low HIV RNA loads observed in the present study in the brains of some demented patients are not concordant with the severity of neurologic dysfunction, suggesting that indirect mechanisms may be more significant than viral burden. Clinico-pathological studies have previously shown that only 50% of AIDS dementia cases are associated with encephalitis (Glass *et al*, 1993). Dementia has not been found to be correlated with neuronal loss in the neocortex of patients with HIV-associated dementia complex (Seilhean *et al*, 1993). High levels of tumor necrosis factor α (TNF-α) mRNAs and of TNF-α expressing cells were shown in the brains of demented patients with AIDS (Wesselingh *et al*, 1993; Seilhean *et al*, 1997).

Our present data show that HIV-1 active replication may not be always sufficient to account for the neuronal injury that ultimately underlies AIDS-associated dementia. Cerebral dysfunction could result from secondary mechanisms, such as immunological factors and cytokine production. A better definition of these secondary events leading to CNS dysfunction will help to develop target therapies for HIV-associated dementia.

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