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Molecular analysis of cerebrospinal fluid: potential for the study of HIV-1 infection of the central nervous system

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The molecular analysis of cerebrospinal fluid (CSF) provides an inestimable tool for the study of HIV infection of the central nervous system (CNS). Current nucleic acid amplification techniques enable the measurement of CSF HIV-1 RNA levels which can be predictive of HIV-associated neurological damage. CSF HIV-1 RNA levels do not necessarily correlate with the corresponding plasma levels, thus supporting the possibility of an intrathecal virus production, i.e., from brain macrophages. However, in early stages of HIV infection, as well as during some opportunistic CNS diseases, CNS or CSF infiltrating lymphocytes might be the main source of CSF virus. A drastic decrease in CSF viral load is usually observed along with a decrease in plasma levels in patients receiving highly active antiretroviral therapy (HAART), with durable suppression of CSF viral load over months. However, during the first weeks of therapy, the dynamics of response may differ in the CSF as compared to plasma, again suggesting that virus replication may be compartmentalised in the CSF. A number of mechanisms are likely to be involved in the response to therapy in CSF, including among the others the trafficking of cell populations supporting viral replication between blood, CNS and CSF, and the role of the anatomical brain barriers in limiting the access of antiretroviral drugs into the CSF. A potential risk associated with compartmentalisation of HIV infection is of an incomplete suppression of virus replication in the CSF, thus creating the ground for local development of anti-HIV drug resistance. In order to assess this occurrence, long-term studies of viral load and genotypic analyses on paired CSF and plasma will be necessary and these will also help elucidate the complex interrelationship between viral replication in these compartments. *Journal of NeuroVirology* (2000) **6,** S95–S102.

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Introduction

Over recent years, the molecular analysis of cerebrospinal fluid (CSF) has been successfully applied to the study of HIV-associated CNS diseases, whether these are opportunistic infections or directly caused by HIV itself. The use of polymerase chain reaction (PCR) and other nucleic acid amplification techniques has made it possible to identify and quantify CSF HIV-DNA or RNA in a reliable manner, and the application of PCR-based sequencing techniques has provided a new and rapid means for HIV genotype analysis.

The assessment of plasma HIV-RNA load has led to revolutionary advances in our understanding of viral dynamics within the human host, and currently represent one of the most important tools for monitoring the response to antiretroviral therapy (Piatak *et al*, 1993; Mellors *et al*, 1997; Perelson *et al*, 1996). More recently, CSF HIV-RNA concentrations have also been assessed in order to evaluate their potential as a marker for the clinical management of patients with HIV-related neurological complications and as a means of improving our

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knowledge of the virus-related events that take place within the central nervous system (CNS).

Although CSF analyses are traditionally used to study the nervous system, the results do not always exactly reflect the events occurring in the brain. One important limitation is the fact that the CSF may contain molecules originating from the blood, which may be relevant in the case of systemic infections, such as HIV infection, that may persist for a number of years. Nevertheless, CSF analyses do provide a unique means of studying the CNS compartment *in vivo*. From a practical point of view a lumbar puncture is routinely used as part of the diagnostic work-up of patients with neurological problems; because it is not over-invasive, and the fact that it can be repeated over time makes it possible to undertake sequential analyses in the same patient.

CSF HIV-1 RNA load

HIV replication in the CNS is probably one of the most important determinants of HIV neurological damage, which is why the measurement of CSF viral levels is considered potentially helpful in the study of HIV-related CNS disease. In order to assess the clinical significance of HIV-RNA replication in the CSF, a number of studies have investigated the correlations between CSF HIV-RNA levels and disease stage, neurological status and neuropathology findings (Brew *et al*, 1997; McArthur *et al*, 1997; Ellis *et al*, 1997; Bossi *et al*, 1998; Cinque *et al*, 1998a; Hengge *et al*, 1998; Di Stefano *et al*, 1998; Robertson *et al*, 1998).

It has been shown that CSF HIV-RNA levels are higher in patients with more advanced stage of HIV infection or low CD4 cell counts than in those at earlier stages or with higher CD4 cell counts (Ellis *et al*, 1997; MacArthur *et al*, 1997). With regard to HIV-induced neurological disease, the CSF viral load has been found to be higher in patients with HIV-associated dementia (HIV-D) than in those with no or milder neurological symptoms (Brew *et al*, 1997; MacArthur *et al*, 1997; Ellis *et al*, 1997). In order to assess whether CSF HIV replication may be predictive of brain damage, HIV-1 RNA levels have also been evaluated in patients classified on the basis of histopathological criteria: i.e., the presence of brain lesions associated with HIV infection, or the demonstration of HIV in the brain by means of immunocytochemical techniques (HIV encephalitis). As expected, a correlation was also found between HIV RNA levels and the presence and severity of HIV encephalitis (Cinque *et al*, 1998a). Certain opportunistic CNS infections, however, have also been shown to affect CSF HIV RNA levels. Elevated virus concentrations have in fact been found in patients with lymphocytic meningitis, such as cryptococcosis or tuberculous meningitis (Brew *et al*, 1997; Morris *et al*, 1998). These conditions are characterised by a marked inflammatory meningeal infiltration and a correlation has been demonstrated between the number of lymphocytes in the CSF and CSF viral load.

Interesting insights concerning HIV replication in the brain have been provided by studies of HIV RNA concentrations in paired CSF and plasma specimens (Brew *et al*, 1997; Ellis *et al*, 1997, McArthur *et al*, 1997; Cinque *et al*, 1998a; Hengge *et al*, 1998; Garcia *et al*, 1999). Although median or mean plasma levels are generally found to be higher than the correspondent concentrations in CSF, the latter can be higher in individual patients. Furthermore, a correlation between CSF and plasma levels has been observed in some studies, but it has not been confirmed by others. Such a correlation is more likely to be observed in asymptomatic patients, whereas CSF and plasma titres are more often dissociated in patients with a more advanced disease stage (MacArthur *et al*, 1997; Cinque *et al*, 1998a; Garcia *et al*, 1999). Finally, several studies have correlated the CSF HIV RNA concentrations with the status of the blood-brain barrier, showing that the presence of a barrier damage, usually calculated using the albumin-globulin ratio, does not seem to affect substantially the CSF viral load (Brew *et al*, 1997; MacArthur *et al*, 1997; Morris *et al*, 1998).

Following these observations, two different types of considerations can be done. The first relates to the interrelationship between viral replication in the CSF, CNS and plasma. In this regard, it has to be considered that the brain is structured into compartments, i.e., intracellular space, extracellular space, blood and CSF. Barrier systems exist between these compartments, consisting of the blood-brain barrier, at the brain capillary site, the blood-CSF barrier, at the choroid plexus, and the CSF-brain barrier, at the lining of the ventricular and brain surface. Whereas the blood-brain and the blood-CSF barriers are sealed by tight junctions (between brain capillary endothelial cells and choroid epithelial cells, respectively), no close anatomical barrier actually exists between CSF and brain extracellular fluid, where the ependimal cells form only a loose interface. CSF is mainly produced across the choroid plexus capillaries; once formed, it moves by bulk flow into the subarachnoidal space and around the brain surface, and, finally, exits into the venous system (recently reviewed by Grootius and Levy, 1997).

On the basis of this model, at least three possible sources of HIV RNA in the CSF can be hypothesised. First, the brain macrophage-microglial cells: according to this hypothesis, the virus produced by these cells is released into the extracellular space, from where it flows into the CSF. This possibility is supported by the possible dissociation between CSF and plasma viral loads (Brew *et al*, 1997; Ellis *et al*, 1997; McArthur *et al*, 1997; Cinque *et al*, 1998a), and by the presence of high CSF HIV-RNA levels in

patients with productive HIV infection, i.e., HIV encephalitis (MacArthur *et al*, 1997; Cinque *et al*, 1998a). Second, blood lymphocytes may be a source of HIV RNA in the CSF: virus particles or genomes that are produced in blood could enter the CSF, either directly through the blood-CSF barrier or indirectly through the blood-brain barrier. This hypothesis is supported by the experimental evidence that inert virus-sized particles may leak into the CSF when very large amounts are injected into the blood (Mims *et al*, 1995). Furthermore, CSF infection is likely to be a prerequisite for the development of certain ventriculo-encephalitides, e.g., those caused by cytomegalovirus or other herpesviruses (Cinque *et al*, 1997a). On the other hand, the large experience collected using CSF PCR in viral diseases other than those caused by HIV, including blood-borne CNS diseases, indicate that, in most of the cases, a virus is not detectable in the CSF unless productive infection takes place in the CNS (Cinque *et al*, 1997b). Third, HIV RNA in the CSF may originate from brain, meningeal or CSF lymphocytes: in particular situations, HIV-infected lymphocytes could pass the blood-CSF or the blood-brain barriers and virus be produced *in situ*. In support of this hypothesis is the correlation between CSF mononuclear cell counts and CSF viral load (Martin *et al*, 1998) and the observation of high CSF HIV RNA concentrations in patients with opportunistic brain infections characterised by the presence of CSF infiltrating lymphocytes (Brew *et al*, 1997; Morris *et al*, 1998). However, the relationship between opportunistic CNS diseases and CSF viral load can be even more complex, since *in vitro* studies have shown that HIV replication can be enhanced by opportunistic pathogens (Pettoello-Mantovani *et al*, 1992; Lederman *et al*, 1994).

A second order of considerations concerns the potential of CSF viral load to predict HIV-related neurological disease. One of the common features of all of the above mentioned studies has been a certain degree of overlapping of HIV-RNA levels between patients with or without HIV-associated dementia, and those with or without HIV encephalitis. Higher CSF HIV loads have been found in patients with HIV-D and/or HIV encephalitis, but significant concentrations have also been detected in patients without any HIV-related neurological disease. On the other hand, low HIV-RNA levels have been detected in both patient groups, with or without HIV-induced neurological disease. On the practical side, the use of CSF viral load for predicting HIV encephalitis appears to be a fairly specific but low sensitive marker, with estimated sensitivity and specificity values of 59 and 93%, respectively, when a cut-off value of 32 000 copies/ml was used (Cinque *et al*, 1998a). Overall, these observations can be interpreted in the light of the previous observations regarding the origin of CSF viral RNA. However, they also indicate that HIV-associated neurological disease is not consistently associated with HIV replication in the CNS or CSF, supporting the view that factors other than virus load may contribute to the development of HIV-related lesions or to the neurocognitive impairment (Nottet and Gendelman, 1995).

In this regard, a number of cytokines and chemokines, neurotoxins and, more in general, immune activation markers have also been studied in the CSF, providing useful information on the mechanisms involved in HIV-related neurological damage (Nottet and Gendelman, 1995). Some of these molecules have also been proposed as diagnostic markers of HIV-related neurological conditions. Actually, elevated levels of some of these markers, including among the others neopterin, β2 microglobulin, quinolinic acid, monocyte chemotactic protein-1, have been found in association with HIV-related dementia or encephalitis (Heyes *et al*, 1991; Brew *et al*, 1996, Cinque *et al*, 1998b; Kelder *et al*, 1998). However, their role in the diagnostic armamentarium of HIV-related conditions is not clearly defined, and also the intriguing possibility of their use in combination with CSF viral load remains to be evaluated.

In summary, the data provided by CSF viral load studies show that, although HIV replication in the CSF may reflect productive CNS infection, CSF HIV RNA levels may in some cases merely reflect plasma concentrations. From a practical point of view, assessment of CSF viral load may be helpful in the clinical management of patients with neurological diseases, however the results need to be interpreted cautiously, keeping into account the individual clinical context, as well as the results from plasma viral load and CSF differential cell count analyses. A better knowledge of the significance of CSF viral load, including its limitations, will also provide an extremely precious tool for studying the relationship between viral replication in the blood and brain compartments, in the perspective of a clearance of HIV infection from the reservoirs.

The effects of antiretroviral therapy on CSF viral load

The antiretroviral drugs currently approved for the treatment of HIV-infected patients include nucleoside reverse transcriptase inhibitors (NRTIs)(Zidovudine or AZT, zalcitabine or ddC, didanosine or ddI, stavudine or d4T, lamivudine or 3TC, abacavir), non nucleoside reverse-transcriptase inhibitors (nNRTIs) (nevirapine, delavirdine, efavirenz), and protease inhibitors (indinavir, nelfinavir, ritonavir, saquinavir, amprenavir). It has been shown that combination treatments containing three or four drugs active against both RT and protease (highly active antiretroviral therapy, or HAART) dramatically decrease plasma HIV-RNA levels, increase CD4 cell counts, and reduce patient mortality. Following treatment, the virus often becomes

undetectable in plasma, although it may persist in certain tissue and cell compartments, or 'sanctuary' sites. In addition to the lymphoid organs and latently infected CD4 cells (Perelson *et al*, 1997; Finzi *et al*, 1997), the CNS is theoretically one of these protected sites, in which HIV-1 replication may occur relatively independently of systemic events.

CSF studies have been undertaken in patients receiving antiretroviral drugs in order to assess the virological response in this compartment (Table 1). A recent retrospective study of neurologically asymptomatic patients receiving monotherapy regimens has demonstrated a significant decrease in viral load after a few months of AZT or ddI treatment (Gisslen *et al*, 1998a). The association of two NRTIs (3TC plus AZT or d4T) in neurologically asymptomatic patients also leads to a significant decline in CSF HIV-1 levels, which become undetectable in virtually all treated patients after 3 months of therapy (Foundraine *et al*, 1998). Ever since the introduction of potent antiviral combinations, a number of reports have documented a rapid decrease in CSF viral loads in patients at different disease stages, including neurologically asymptomatic patients, patients with mild or severe HIV-related symptoms, and patients with opportunistic brain diseases (Gisslen *et al*, 1998a, b; Iftimovici *et al*, 1998; Staprans *et al*, 1999; Eggers *et al*, 1999).

A clinical neurological response has also been documented in patients receiving antiretroviral therapy. Early studies have described an improvement of both neurological symptoms and neuropsychological tests following AZT administered as monotherapy (Schmitt *et al*, 1988; Sidtis *et al*, 1993). Furthermore, a decline of HIV-associated dementia incidence and a reduced frequency of HIV-related neuropathological lesions was shown following the introduction of this drug (Portegies *et al*, 1989; Vago *et al*, 1993). More recently, improvements of neurological or neuropsychological functions have been shown in patients receiving double NRTI therapy (AZT and ddI) or HAART (Henry *et al*, 1998; Sacktor *et al*, 1999; Tozzi *et al*, 1999). Although results from systematic studies assessing both neurological functioning and CSF viral load are not yet available, it is reasonable to hypothesise and there is initial evidence in support showing that virological and clinical parameters of HIV brain infection can respond concomitantly to antiretroviral therapy (Gendelman *et al*, 1998).

Among HAART treated patients differences in the dynamics of the CSF and plasma responses of individual patients have been observed: the decrease in plasma and CSF HIV-RNA load is similar in some patients, but others show a slower decline in the CSF than in plasma, once again suggesting that HIV infection may be variably compartmentalised in the CSF and, possibly, in the CNS (Iftimovici *et al*, 1998, Staprans *et al*, 1999, Cinque *et al*, 2000; Letendre *et al*, 2000). In theory, virus originating from lymphocytes could have been present in the CSF of the patients with similar CSF and plasma responses. In contrast, the lower CSF response in some patients could indicate virus production by local macrophages: these cells, in fact, support virus replication at a rate which is different from that observed in lymphocytes (Price and Staprans, 1997). Furthermore, penetration of antiretroviral drugs through the brain barriers, as well as their uptake and intracellular metabolism in macrophages may theoretically limit drug efficacy in the brain (O'Brien *et al*, 1994).

If HIV may replicate in the CNS relatively apart from other body sites, it follows that virological escape may occur in the brain despite a systemic virological response. However, on the basis of our own and others' CSF studies in patients treated with antiretroviral combinations for up to 3 years, virological escape in the CNS appears to be only a rare occurrence (Pialoux *et al*, 1997). Nevertheless, longer-term follow-up observations involving larger patient groups appear to be necessary in order to define the real importance of the CNS as a viral reservoir in patients treated with antiretroviral combinations.

Assessing genotype resistance in the CSF

The high rate of HIV-1 replication, coupled with the high error rate of HIV-1 reverse transcriptase, which has no proof-reading ability, allow the continuous generation of different genetic variants *in vivo*. Nucleotide mutations may lead to no change, a nonsense codon, or a codon specifying a different aminoacid. In the latter case, changes may occur at critical sites and may alter protein structure and function. For resistance to an antiretroviral agent to occur, the target enzyme, i.e., RT or protease, must modify but preserve its function in the presence of the drug. The selective pressure of antiretroviral therapy gives drug-resistant mutants a competitive advantage and they eventually come to represent the dominant variant. In general, genotypical changes correlate with phenotypical measurements of sensitivity, and the resistance mutations of the RT and protease genes are well known for the principal classes of the currently available drugs (Hirsch *et al*, 1998).

Although the aim of antiretroviral therapy is to control the rate of viral replication, a number of factors may lead to an incomplete suppression of the viral load and the greater development of drug resistant mutants. These factors include patient adherence, individual drug absorption and metabolism, the intrinsic rate of viral replication and mutation, and the existence of cell and tissue reservoirs. The presence of functional and anatomical barriers in the CNS provides a tissue reservoir in which virus replication may be incompletely suppressed, thus creating the chance for the

Table 1 CSF viral load and antiretroviral therapy: main findings from the principal published studies.

Reference	*Treatment*	*Number of patients*	*Patient neurological status*	*Treatment status*	*CD4+/μl (range)*	*Treatment length*	*Baseline CSF HIV-1 RNA (median log^{10} copies/ml)*	*CSF HIV-1 RNA changes (median log^{10} copies/ml)*	*Baseline plasma HIV-1 RNA (median log^{10} copies/ml)*	*Plasma HIV-1 RNA changes (median log^{10} copies/ml)*
Gisslen *et al,* 1997[a]	AZT	13	Neurologically asymptomatic	Naive or ddl-experienced	34–425	4–13 months	3.95	−1.05	4.21	+0.05
	ddl	8	Neurologically asymptomatic	Naive or AZT-experienced	36–302	3–12 months	3.92	+0.13	4.24	−0.53
Foudraine *et al,* 1998	AZT+3TC	11	Neurologically asymptomatic	Naive	210–500	12 weeks	4.64	HIV-1 RNA undetectable in all	4.81	−1.56
	3TC+d4T	17	Neurologically asymptomatic	Naive	245–430	12 weeks	4.20	HIV-1 RNA undetectable in all	4.98	−2.27
Gisslen *et al,* 1998a	AZT+3TC+IDV	10	Neurologically asymptomatic	Naive	44–484	1.4–6.1 months	3.67	HIV-1 RNA undetectable in all[b]	4.70	1.61
Gisslen *et al,* 1998b	AZT+3TC+IDV	6	Neurologically asymptomatic	Naive	Not provided	12.4–15.7 months	3.67	HIV-1 RNA undetectable in all but one patient (2.07 log^{10} copies/ml)[b]	Not provided	Not provided
Staprans *et al,* 1999	HAART	13	HIV-D or neurologically asymptomatic	Naive or ART experienced	3–1140	<11 days	4.47	−0.13 per day	5.10	−0.16 per day
	HAART	11	HIV-D or neurologically asymptomatic	Naive or ART experienced	45–295	4–38 weeks	4.92	−2.43	5.10	2.43
Eggers *et al,* 1999	HAART	15	HIV-D, CNS Ols, or PHI	Naive or ART experienced	10–580	5–24 days	3.65	−1.37[c]	5.02	−1.65

[a]In this study mean values were considered; [b]An 'ultrasensitive' assay, with a lower detection limit of <20 copies/ml was used; [c]Two patients in whom a rise of CSF viral load was observed were not included in the analysis. ART: antiretroviral therapy; AZT: zidovudine; ddl: didanosine; d4T: stavudine; HAART: highly active antiretroviral therapy; HIV-D: HIV-associated dementia; IDV: indinavir; Ol: opportunistic infection; 3TC: lamivudine.

development of distinct resistant mutants at this level.

A few studies have addressed the question as to whether there may be different resistant mutations between the CSF and plasma of patients receiving antiretroviral therapy (Wildemann *et al* 1993; Di Stefano *et al*, 1995; Wong *et al*, 1997). The early studies were performed by sequencing the RT gene on viral DNA obtained directly from CSF and blood or CSF/blood isolates and, although the overall number of patients was low, different resistant mutations in the two compartments were demonstrated in some (Wildemann *et al,* 1993; Di Stefano *et al*, 1995). Some differences between CSF and plasma resistance patterns have also more recently been shown with regard to the 3TC-induced mutation at codon 184 and to mutations detectable by a commercial probe hybridization assay (Chien *et al*, 1999; Cunningham *et al*, 1998). We sequenced the whole protease gene and a large part of RT in paired CSF and plasma specimens, and assessed the presence of resistant mutations in the two compartments. Following analysis of CSF and plasma pairs from 50 patients who had experienced RTIs but not PIs, the RT genotype resistance patterns were different between the two compartments in approximately one quarter of the patients, whereas no primary PI mutations were found. In approximately half of the cases in which CSF and plasma mutations were different, the CSF harboured virus containing mutations that were not identifiable in plasma, thus suggesting that drug-resistant mutants can actually be selected *in vivo* in this compartment (our unpublished data). The comparison of viral sequences and the virological response to antiretroviral therapy in paired CSF and blood specimens is the most obvious way of continuing such studies and will probably help define the role of HIV infection of the CNS in the era of potent antiretroviral combination therapies.

Conclusions

The data collected so far argue for the possibility that the dynamics of HIV-1 differ in CSF and blood, despite the fact that these two compartments appear to be highly interactive at least in certain stages of HIV infection. Although it is possible that resistant virus is selected in the CNS, CSF studies suggest that the antiretroviral combinations that are effective in controlling HIV replication in the blood are usually also efficacious in the CNS. However, some aspects of the relationship between viral dynamics and antiretroviral treatment remain to be clarified. In this regard, the application of current sequencing techniques to the study of CSF provides an invaluable tool for HIV-1 genotype analyses in patients receiving antiretroviral treatments.

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