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Macrophage chemoattractant protein-1 levels in cerebrospinal fluid correlate with containment of JC virus and prognosis of acquired immunodeficiency syndrome–associated progressive multifocal leukoencephalopathy

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In the highly active antiretroviral therapy (HAART) era, the role of the inflammatory response in acquired immunodeficiency syndrom (AIDS)-related progressive multifocal leukoencephalopathy (PML) remains controversial. In this study, JC virus DNA load and levels of cytokines were determined in cerebrospinal fluid (CSF) from 32 human immunodeficiency virus (HIV)-1–infected patients with confirmed PML who underwent HAART; cytokines were also measured in 12 HIV-positive controls. Predictors of survival were analyzed by Cox's models. Macrophage chemoattractant protein (MCP)-1 levels were significantly higher in PML patients than in controls (mean $\pm$ SD, 2.45 $\pm$ 0.64 versus 1.32 $\pm$ 0.64 $\log_{10}$ pg/ml, $P < .0001$). In PML patients, the higher concentration of MCP-1 correlated with lower JC viral load ($r = -.405$, $P = .036$). Higher concentrations of MCP-1 in CSF were associated with longer survival on HAART after adjusting for CD4 counts (for each $\log_{10}$ pg/ml higher, hazard ratio for death 0.28, 95% confidence interval 0.08–1.00). Predictors of shorter survival were lower baseline CD4 counts, higher JCV DNA concentrations, lower Karnofsky, and no prior HAART exposure. These results showed that higher CSF levels of MCP-1, an inflammatory cytokine, were correlated with better prognosis in HAART-treated patients with PML. *Journal of NeuroVirology* (2005) **11,** 219–224.

Keywords: AIDS; cerebrospinal fluid; JC virus; MCP-1; progressive multifocal leukoencephalopathy; viral load

Introduction

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease of the human central nervous system with a poor prognosis and a high case fatality rate (Brooks and Walker, 1984). It is associated with lytic infection of oligodendrocytes by the poliomavirus JC and occurs mainly in patients with underlying immunodeficiency (Berger and Concha, 1995). In autoptic case series from acquired immunodeficiency syndrome (AIDS) patients, PML prevalence ranges between 5% and 8%; it represents the third most frequently notified neurological complication among human immunodeficiency virus (HIV)-1–infected patients (Antinori *et al*, 2003). After the introduction of highly active antiretroviral

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Informed consent was obtained from subjects in accordance with guidelines of the local institutions where the study was conducted.

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therapy (HAART) into clinical practice, the incidence of many AIDS-related opportunistic infections has dramatically declined, but PML figures show a less significant reduction (Palella *et al*, 1998). Several observational studies report improved survival in PML patients treated with HAART (De Luca *et al*, 2001; Gasnault *et al*, 2001; Berenguer *et al*, 2003). Nevertheless, the mortality of PML remains approximately 50% and the survivors show a stabilization of their neurological deficits, often resulting in a severe chronic disability (De Luca *et al*, 1998, 2001; Gasnault *et al*, 1999). Furthermore, some cases of PML developing after the onset of HAART show features of an immune reconstitution syndrome and are sometimes associated with an unfavorable clinical outcome. On the other hand, cases of inflammatory PML have been described showing a favorable outcome in association with JC virus–specific T-cell responses (Du Pasquier and Koralnik, 2003; Gasnault *et al*, 2003). PML survival may be affected by several factors: those identified are virological (De Luca *et al*, 1999, 2000), clinical (Antinori *et al*, 2001, 2003) and immunological (Gasnault *et al*, 2003; De Luca *et al*, 2000). Several studies indicate that in HIV-1–infected patients exposed to HAART, cerebrospinal fluid (CSF) levels of inflammatory cytokines are increased (Gisolf *et al*, 2000).

Macrophage chemoattractant protein (MCP)-1 is an inflammatory and chemotactic cytokine that is produced by activated HIV-1–infected macrophages and is associated with recruitment of monocytes in the central nervous system (McManus *et al*, 2000). Elevated MCP-1 levels have been detected in patients with HIV-associated dementia (Conant *et al*, 1998) and encephalitis due to HIV-1 and cytomegalovirus (Cinque *et al*, 1998; Bernasconi *et al*, 1996), suggesting that this chemokine is involved in inflammatory processes in the brain of infected individuals. Tumor necrosis factor alpha (TNF-alpha) is a cytokine produced by monocytes and macrophages and plays a role in the pathogenesis of many inflammatory diseases (Vilcek and Lee, 1991). Regulated upon activation normal T cell expressed and presumably secreted (RANTES), a beta chemokine, is the natural ligand of HIV-1 coreceptor CCR5 and a potent soluble inhibitor of HIV-1 infection of brain cells, which acts by blocking the binding of HIV gp-120 to this co-receptor on the surface of glial cells (Nardese *et al*, 2002; Kitai *et al*, 2000). The aim of our study was to evaluate the CSF levels of these cytokines in patients with AIDS-related PML and their relation to other immunologic and virologic markers and to patients survival after HAART.

Results

Patients characteristics

We studied 44 HIV-1–infected patients: 70% were males and their median age was 37 years (standard deviation [SD], ±7.3). Thirty-two patients had confirmed PML: JC virus DNA was detected in CSF in 31 of 32 cases and histology confirmed the diagnosis in 4 cases (three patients had both virological and histological diagnosis). In PML cases, the median CD4 count was 48.5 cells/μl (interquartile range [IQR], 16.5–124), the mean CSF JC viral load was 4.74 $\log_{10}$ copies/ml (range 2.70–11.03), the median HIV-1 RNA was 4.27 $\log_{10}$ copies/ml (IQR, 2.97–5.20) in plasma and 2.96 $\log_{10}$ copies/ml (IQR, 1.89–3.84) in CSF. Antiretroviral treatment history revealed that 9 out of 32 persons with PML had been exposed to a protease inhibitor (PI)-based HAART for a median of 9 weeks (IQR, 5–40) before neurological onset. After PML diagnosis, all patients were treated with a PI-based HAART and 12 persons also received cidofovir for a median of 7.5 intravenous cycles (range, 2–30).

Twelve HIV-1–infected patients selected to lumbar puncture were used as controls. Reasons for performing diagnostic lumbar punture was the onset of some neurological signs or symptoms in 11 cases (persistent headache in 6 cases and dizziness with psichomotor slowering in 5; in all cases fever was present). Another patient underwent disease staging of a systemic non-Hodgkin's lymphoma. The median CD4 cell count of these controls was 27.0 cells/μl (IQR, 21–87), not significantly different from that of patients with PML. The HIV RNA concentrations in plasma and CSF (the latter available in 5 of 12 patients) did not differ from those of PML cases. Their diagnostic procedure did not result in the detection of any opportunistic infection or brain tumor. Neuroradiological exams were negative and, although formal neuropsichological testing was not performed, patients did not present major cognitive impairment. CSF samples obtained from these patients resulted negative for JC virus DNA, Epstein-Barr virus DNA, cytomegalovirus DNA, herpes simplex virus DNA, and varicella-zoster virus DNA by several polymerase chain reaction (PCR) assays. Neurological symptoms either resolved spontaneously with the resolution of fever or were attributed to the use of opiates or benzodiazepines.

Cerebrospinal fluid cytokine concentrations

The MCP-1 levels were obtained in 31 out of 32 samples collected from PML patients and 12 collected from controls. Overall, the median concentration of MCP-1 was 360 pg/ml (IQR, 14–590 pg/ml). TNF-alpha was detected in samples from 28 patients with PML and from 11 controls: the median level of this cytokine was 45 pg/ml (IQR, <10–200 pg/ml). CSF levels of RANTES were measured in 27 samples from PML subjects and 11 samples from controls and the overall median concentration was 100 pg/ml (IQR, 24–216 pg/ml).

Figure 1 shows CSF concentrations of cytokines in PML patients and in HIV-1–infected controls. CSF samples collected from PML patients showed significantly higher levels of MCP-1 (mean ± SD, 2.45 ±

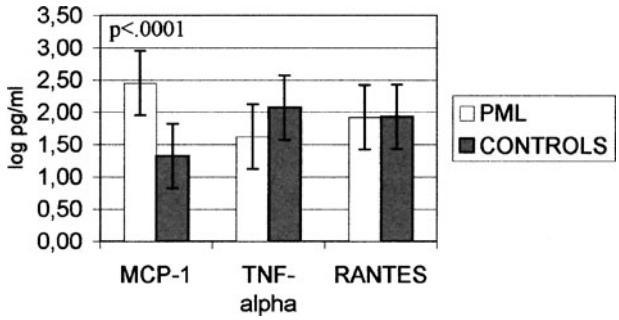

Figure 1 Cytokines concentrations in CSF of AIDS-associated PML patients and HIV-1–infected controls. Columns indicate means, vertical bars with whiskers indicate standard deviations.

0.64 $\log_{10}$ pg/ml) than those collected from controls (1.32 ± 0.64 $\log_{10}$ pg/ml, $P < .0001$). CSF levels of TNF-alpha were not significantly lower in PML patients (mean ± SD, 1.62 ± 0.83 $\log_{10}$ pg/ml) than in controls (2.07 ± 0.82 $\log_{10}$ pg/ml; $P = .14$). Mean CSF levels of RANTES were similar in both groups of patients (1.92 ± 0.55 $\log_{10}$ pg/ml in cases and 1.93 ± 0.51 $\log_{10}$ pg/ml in controls, $P = .97$).

Correlations of cytokine levels with immunological and virologic markers

In the 32 patients with PML, we analyzed whether cytokine concentrations were correlated with each other and whether there was any correlation with CD4 counts, CSF concentration of JC virus DNA or HIV-1 RNA, and with prior treatment history.

There was a positive linear correlation between MCP-1 and TNF-alpha levels in CSF ($r = .52$, $P = .024$), although the latter were consistently lower and often undetectable. No other association between cytokine concentration was detected.

After stratifying PML patients by level of CD4 cell count, we observed that CSF concentrations of TNF-alpha were higher in subjects with lower CD4 counts (mean ± SD, 1.80 ± 0.92 log pg/ml in patients with CD4 <100 cells/μl versus 1.00 ± 0 log pg/ml in patients with CD4 >100 cells/μl; $P = .012$). On the other hand, there was no association between CD4 counts and CSF levels of MCP-1 ($P = .99$) or RANTES ($P = .33$).

We found a significant negative correlation between the CSF levels of MCP-1 and the JC viral load ($r = -.405$, $P = .036$) (Figure 2), whereas TNF-alpha and RANTES did not correlate with JC virus concentrations. There were trends towards a positive linear association between MCP-1 concentrations and HIV-1 viral load levels in CSF ($r = .41$, $P = .08$). On the contrary, no correlation was found between TNF-alpha and, RANTES levels and HIV-1 RNA concentrations.

Although MCP-1 levels did not differ in PML patients who had been previously exposed to HAART when compared to those who were not, in the nine subjects previously exposed to HAART, MCP-1 showed a trend towards a positive correlation with the time of prior HAART exposure ($r = .61$, $P = .07$).

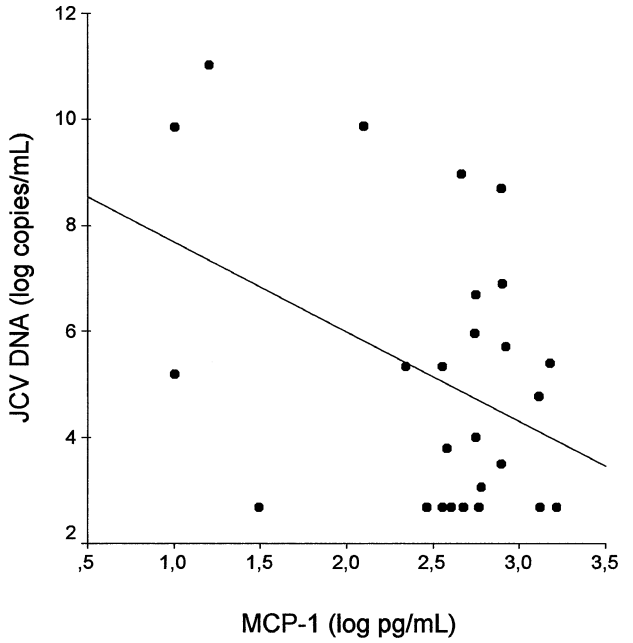

Figure 2 Plot indicating the linear correlation between cerebrospinal fluid concentrations of macrophage chemoattractant protein (MCP)-1 and JC virus DNA in HIV-infected PML patients ($n = 27$, $r = -.405$, $P = .038$). Dots indicate individual values, the line indicates the linear regression.

In the 12 controls, we could not find any association among cytokine levels or between any cytokine concentration and CD4 counts or HIV RNA.

Patients survival and its predictors

Despite HAART given to all patients after PML diagnosis, there were 24 PML-related deaths during the follow-up. Using the Kaplan-Meier method, the cumulative proportion of patients surviving at 6 months was 0.49 (0.41 at 1 year).

At univariate analysis, factors that predicted a higher hazard ratio of death were lower Karnofsky score, higher JCV DNA levels in CSF, lower CD4 cell counts, and absence of prior HAART exposure (Table 1). Of interest, in 23 PML patients with less than 100 CD4 cells/μl, higher CSF concentration of MCP-1 were associated with a longer survival.

Noteworthy, in a multivariable Cox's model in the total set of PML subjects, after adjusting for baseline CD4 counts, MCP-1 levels were independently predictive of longer survival (for each $\log_{10}$ pg/ml higher, hazard ratio for death 0.28, 95% confidence interval 0.08–1.00, $P = .05$).

Discussion

In the present study, CSF samples collected from HIV-1–infected PML patients showed significantly higher

Table 1 Predictors of survival in PML patients ($n = 32$): univariate analysis

Variables	*Hazard ratio for death (95% CI)*	P *value*
Baseline CD4+ counts (per $\log_2$ cells/μl increase)	0.76 (0.59–0.97)	.031
JC virus DNA concentration in CSF (per $\log_{10}$ copies/ml increase)	1.48 (1.17–1.86)	.001
Baseline Karnofsky (per 10 points increase)	0.74 (0.58–0.94)	.013
HAART exposure prior to PML onset	0.42 (0.17–1.02)	.053
MCP-1 levels in CSF (per of $\log_{10}$ pg/ml increase)	0.42 (0.11–1.60)	.214
MCP-1 levels in CSF* (per of $\log_{10}$ pg/ml increase)	0.27 (0.08–0.96)	.040

*In patients with less than 100 CD4 cells/μl ($n = 23$).
PML, progressive multifocal leukoencephalopathy; CI, confidence interval; CSF, cerebrospinal fluid; HAART, highly active antiretroviral therapy; MCP-1, macrophage chemoattractant protein type 1.

levels of MCP-1 but not TNF-alpha or RANTES as compared to HIV-1–positive controls with comparable CD4 counts. The MCP-1 levels tended to be higher among PML patients with a longer prior exposure to HAART. TNF-alpha and MCP-1 concentrations were positively correlated in PML patients but TNF-alpha showed lower and often undetectable levels. We found a negative correlation between the CSF concentrations of MCP-1 and the concentrations of JC virus in this fluid. Higher CSF levels of MCP-1 were associated with longer survival in patients with CD4 counts lower than 100 cells/μl. Multivariable analysis confirmed the association of MCP-1 with longer survival after adjusting for CD4 counts. On the contrary, CSF levels of RANTES and TNF-alpha were not associated JC virus concentration nor with the survival of PML patients. Findings from this study suggest that some type of inflammatory reaction that occur in selected HIV-1–infected PML patients, such as that represented here by the elevated CSF levels of MCP-1, could be beneficial for the prognosis of the disease. This reaction may be in part induced by HAART exposure, it is negatively correlated with JC virus load and, in individuals with low baseline CD4 counts, may predict longer survival. Although higher CD4 cell counts are an important predictor of survival in PML as shown here and by others (Berenguer *et al*, 2003), our findings suggest that MCP-1 levels in CSF can usefully identify patients with better prognosis among those with low CD4 counts.

JC virus–specific immune responses have been demonstrated to favorably correlate with disease prognosis (Du Pasquier and Koralnik, 2003; Gasnault *et al*, 2003). We speculate that, given the inverse correlation with JC viral load, the higher MCP-1 levels might be associated to a JC virus–specific immune response. In alternative, MCP-1 production might simply reflect a concomitant activation of monocyte-derived cells as a result of a reaction that might include, at least in part, virus-specific immune activation. In a single case of our series, an inflammatory infiltrate, mainly composed of macrophages, was detected by histopathological examination of brain biopsy: this case had high levels of MCP-1 in CSF (680 pg/ml) and details have been published elsewhere (Di Giambenedetto *et al*, 2004).

Recently, MCP-1 has been associated with a neuroprotective effect against tat or *N*-methyl-*D*-aspartate (NMDA)-induced apoptosis (Bruno *et al*, 2000; Eugenin *et al*, 2003). In HAART-treated patients, higher MCP-1 levels correlate with elevation of myoinositol levels (Chang *et al*, 2004) and the latter has been shown to favourably correlate with the prognosis of PML (Katz-Bruell *et al*, 2004).

A limitation of this study is that the exact cell type responsible for MCP-1 production in the central nervous system of PML patients was not investigated. Neverthless another report shows the presence of monocytic infiltrates surrounding PML lesions in cases with concomitant JC virus–specific immune reaction (Miralles *et al*, 2001).

In agreement with other studies, our findings show that immune or inflammatory reactions should be regarded as favorable events in the course of AIDS-associated PML in the majority of patients (Giudici *et al*, 2000; Berger *et al*, 1987). There might be exceptions, in which extremely potent inflammatory reaction may cause irreversible brain damage and death (Cinque *et al*, 2001, 2003).

In summary, in HIV-1–infected PML patients we detected higher levels of MCP-1 in CSF: these were related with lower JC virus levels as well as improved survival. If confirmed by other studies, CSF levels of MCP-1, along with other established prognostic indicators, could be a new, easy-to-detect marker for monitoring the course of AIDS-associated PML in the era of HAART.

Materials and methods

Patients selection

We analyzed HIV-infected patients who underwent a diagnostic lumbar puncture at two infectious diseases referral centers in Rome, Italy, between January 1997 and May 2003. Patients with a virologically and/or a histologically confirmed diagnosis of PML were identified. The virological diagnosis of PML was based on the concomitant presence of a compatible clinical and neuroimaging picture and the detection of JC virus DNA in CSF by PCR, whereas the histological diagnosis was based on the presence of characteristic histopathologic features in the brain tissue, including immunohistochemical detection of JC virus antigens in oligodendrocytes (De Luca *et al*, 2000). After PML diagnosis, all patients underwent HAART.

As controls, other HIV-1–infected patients observed at the same two clinical centers during the study period and without active opportunistic infection or brain tumors were selected by matching upon similar CD4 cell count. The study protocol was approved by the local Ethics Committees and all patients gave written informed consent.

Patients characteristics and survival were abstracted from clinical records. For this study, HAART was defined as any combination of no less than three antiretroviral drugs including a PI.

Virological assays

JCV DNA was extracted from cell-free CSF on resin columns according to the manufacturer's instructions (QUIAMP; Quiagen, Hilden, Germany) and the viral genome was quantified by using the Taq Man real-time PCR technology. The PCR primers PEP-1 and PEP-2 were designed to amplify the large T-antigen region of JC virus (Major *et al*, 1992). The probe JCTAQ1 (TGA TGA TGA AAA CAC AGG ATC CCA ACA CTC) was labelled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine. Each 50-μl PCR mixture contained 2 μl of template, 50 nM concentrations of each primer, 20 nM of probe, and 25 μl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). After 2 min of incubation at 50°C and 30 s of incubation at 95°C for denaturation, the samples were subjected to 45 cycles of PCR. During each cycle the temperature was set at 95°C for 20 s and at 54°C for 1 min. Fluorescence intensity was read automatically during PCR cycling in an I Cycler iQ Multicolor Real Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and the generated real-time data were analyzed with sequence detector (Bio-Rad Laboratories) software. Each specimen was run in duplicate with appropriate positive and negative controls. Four different concentrations of a positive control were used to construct a reference standard curve that was employed to calcluate the exact JC virus DNA concentration in the clinical samples. The detection limit of the PCR assay was of 1 viral DNA copy per reaction, corresponding to 500 DNA copies per milliliter of the clinical sample.

HIV-1 RNA levels were quantified in CSF by reverse transcriptase (RT)-PCR using a commercial kit (Ultra sensitive Amplicor HIV-1 Monitor; Roche Diagnostic, Branchburg, NJ) with a detection limit of 20 to 25 copies/ml. Plasma HIV-1 RNA concentrations were measured using a branched DNA assay with a detection limit of 50 copies/ml (Quantiplex 3.0, Chiron, Emeryville, CA, USA).

Immunological assays

MCP-1, RANTES, and TNF-alpha levels were measured in CSF using commercial assays based on quantitative sandwich immune enzymatic techniques (Quantikine; R&D System, Minneapolis, MN, USA), with a detection limit of 10 pg/ml. The instructions given by the manufacturer were followed throughout the procedure. Peripheral blood CD4+ T-cell counts were determined by standard flow cytometry.

Statistical analysis

Viral copy concentrations in body fluids and cytokines levels were log-transformed before calculations. Differences between continuous variables were analyzed using the Student's *t* test, correlations between continous variables were tested by Pearson's analysis. Survival analysis was performed using Kaplan-Meier curves and Cox's proportional hazards models. All analyses were performed using the Statistica version 5.0 software package (Statsoft, Padua, Italy).

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