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Increased activity of matrix metalloproteinases in the cerebrospinal fluid of patients with HIV-associated neurological diseases

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Matrix metalloproteinases (MMPs) have been identified as mediators of brain injury in HIV-associated neurological diseases. The activity of the 72 kDa gelatinase A (MMP-2) and 92 kDa gelatinase B (MMP-9) was detected by zymography in the cerebrospinal fluid (CSF) of 138 HIV-infected patients (40 with AIDS dementia, 83 with brain opportunistic infections and 15 neurologically asymptomatic), 26 HIV-seronegative individuals with inflammatory neurological diseases (IND) and 12 HIV-seronegative subjects with noninflammatory neurological diseases (NIND). MMP-2 was present in all CSF samples from HIV-seropositive and HIV-seronegative individuals, including those of subjects with NIND. On the contrary, MMP-9 was absent in the CSF of NIND controls, whereas the activity of this MMP was found in the 77–100% of CSF samples from HIV-infected patients, including those with HIV dementia, central nervous system (CNS) opportunistic infections or neurologically asymptomatic subjects. The highest levels of MMP-9 were found in the CSF of patients with cryptococcosis, cytomegalovirus encephalitis and tuberculous meningitis and were comparable with those found in the CSF of HIV-negative patients with multiple sclerosis or meningitis. A significant correlation between CSF MMP-9 activity and CSF cell count was found only in patients with HIV dementia. The increased CSF activity of MMPs capable to degrade components of the extracellular matrix of blood-brain barrier may contribute to the transendothelial migration of virus-infected cells into the CNS and development of HIV-associated neurologic damage. *Journal of NeuroVirology* (2000) **6,** 156–163.

Keywords: matrix metalloproteinases; CSF; AIDS dementia; opportunistic infections; blood-brain barrier

Introduction

HIV-1 infection may be complicated by a variety of neurological syndromes including central nervous system (CNS) opportunistic infections, tumours and, frequently, AIDS dementia complex (ADC), which is directly attributable to HIV (Simpson and Tagliati, 1994). It is known that ADC is associated with increased numbers of activated brain macrophages and microglia which secrete high levels of cytokines and neurotoxins contributing to HIV-1-associated neurologic damage in several ways (Gendelman *et al*, 1997). Pathological studies suggest that alterations of the blood-brain barrier (BBB) may lead to the increased transendothelial migration of immune-activated HIV-infected blood monocytes/macrophages into the CNS (Gendelman *et al*, 1997; Petito and Cash, 1990).

Matrix metalloproteinases (MMPs) or gelatinases are a family of Zn-dependent endopeptidases which degrade components of the extracellular matrix of the BBB such as the basement membrane type IV collagen and proteoglycans (Matrisian, 1990; Woessner, 1994). Perivascular macrophages and other cells, including microglia, secrete MMPs as well as specific tissue inhibitors of metalloprotei-

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nases (TIMPs), which regulate the activity of MMPs. Alterations in the balance between MMPs production and the expression of TIMPs seem to play an important role in the pathophysiology of various inflammatory neurological diseases, such as multiple sclerosis (MS) (Yong *et al*, 1998). In acute MS lesions a 92-kDa type IV collagenase (gelatinase B or MMP-9) have been shown *in situ* (Cuzner *et al*, 1996). In addition, elevated levels of MMP-9 have been found in the cerebrospinal fluid (CSF) of patients with MS and other inflammatory neurological disorders, suggesting a role for these proteinases in determining myelin injury and breakdown of the BBB (Gijbels *et al*, 1992; Paemen *et al*, 1994; Paul *et al*, 1998).

Recently, we demonstrated the presence of a myelin-degrading proteolytic activity in the CSF of HIV-infected patients with ADC and progressive multifocal leukoencephalopathy (PML), in which demyelination was observed by magnetic resonance imaging (MRI) (Liuzzi *et al*, 1994). As assessed by its response to inhibitors, this activity appeared to have the character of a serine protease. On the other hand, MMP-9 was identified in the CSF of HIV-infected patients thus suggesting a role as potential mediator involved in BBB leakage during HIV infection (Sporer *et al*, 1998).

The aim of this paper was to further characterise the proteolytic activity released in the CSF of HIV-infected patients with neurological complications, and to investigate the role of MMPs in HIV-associated neurological damage. Therefore, we measured the levels of the 72 kDa gelatinase A (MMP-2) and 92 kDa gelatinase B (MMP-9) in the CSF of HIV-infected patients with various neurological disorders. The results were compared with those obtained in MS patients and in other HIV-seronegative individuals with infectious and non-infectious neurological diseases.

Results

Matrix metalloproteinase activity in the cerebrospinal fluid

CSF samples from HIV-seropositive and HIV-seronegative individuals, with or without neurological diseases, were analysed by zymography on polyacrylamide gels copolymerised with gelatin to detect the presence of gelatinases.

Two main forms of gelatinases were detectable on gels with an apparent molecular mass of 72 and 92 kDa, corresponding to gelatinases A (MMP-2) and B (MMP-9), respectively. In addition, in some CSF samples two minor bands with gelatinolytic activity with an apparent molecular mass of approximately 117 and 130 kDa, were observed. A representative zymography with the analysis of CSF samples from patients of the different disease categories, analysed in this study, is shown in Figure 1.

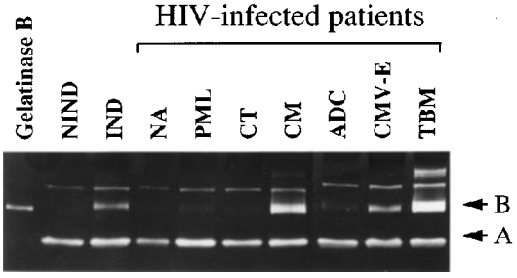

Figure 1 Zymographic analysis of gelatinase A and B activity in the CSF of patients with various neurological disorders. One μl of gelatinase B standard solution or 50 μl of CSF from representative samples from each patient group, according to diagnosis, were loaded into a 10% polyacrylamide gel copolimerised with 0.1% gelatin. Gelatinase activity appears as clear bands on the dark background of the gel. Gelatinase A (72 kDa) and gelatinase B (92 kDa) are indicated by arrowheads. In the upper side of the gel two minor bands of gelatinolytic activity with an apparent molecular mass of approximately 117 and 130 kDa are detectable. The noninflammatory neurological diseases (NIND) sample is from a patient with tension headache. The inflammatory neurologic diseases (IND) sample is from a patient with multiple sclerosis. NA=neurologically asymptomatic; PML=progressive multifocal leukoencephalopathy; CT=cerebral toxoplasmosis; CM=cryptococcal meningitis; ADC=AIDS dementia complex; CMV-E=cytomegalovirus encephalitis; TBM=tuberculous meningitis.

The 72 kDa band was detected in all samples, while the 92 kDa band, corresponding to MMP-9, as indicated by the presence of a standard, was found in 118/138 (87%) of the CSF samples from HIV-infected patients and in 26/26 (100%) samples from patients with IND. MMP-9 activity was not detected in the CSF samples of the NIND group (0/12). Among the HIV-infected patients, the MMP-9 activity was detected in 77% of patients with ADC (31/40), 77% with CT (17/22), 82% with PML (14/17), 100% with CM (14/14), 100% with TBM (9/9), 95% with CMV-E (20/21), and in 87% of HIV^+ NA patients (13/15).

In order to assess whether the absence of MMP-9 in samples from some HIV-infected patients could be due to a more severe immunesuppression, we evaluated the correlation between $CD4^+$ T-cell count in the pooled group of HIV-infected patients and MMP-9 in the CSF. There was no correlation of MMP-9 elevation and $CD4^+$ T cell count (r=0.11; P=0.19) (Figure 2).

Quantitative zymography

In order to quantify MMP-9 (gelatinase B) activity in CSF samples and eliminate the variability due to the different staining intensity between the zymographies, the ratio of gelatinase B to gelatinase A (B/A) was calculated for every sample (Paemen *et al*, 1994; Masure *et al*, 1990). Significantly elevated gelatinase B/A ratios ($P<0.05$) were found in the HIV-infected patients

(mean ± s.e.m., 1.30 ± 0.15) and in the IND patients (3.30 ± 0.46) when compared with the NIND group (0.02 ± 0.01) (Figure 3).

To investigate whether there existed in the group of HIV-infected patients a correlation between neurological diseases and the presence of MMP-9 in the CSF, we analysed MMP-9 levels in each HIV patient group in comparison with the NA subjects. In Figure 4 the distribution of MMP-9 levels in the different groups of HIV-infected patients is illustrated. A statistically significant increase in MMP-9 levels ($P<0.05$) was observed in the CSF of CMV-E (mean ± s.e.m., 1.72 ± 0.322), CM (3.27 ± 0.509), TBM HIV$^+$ (4.729 ± 0.920) patients, when compared with those of NA subjects (0.568 ± 0.205). No significant differences of NA *versus* ADC (0.684 ± 0.133), PML (0.439 ± 0.126) and CT (0.374 ± 0.06) patients were found.

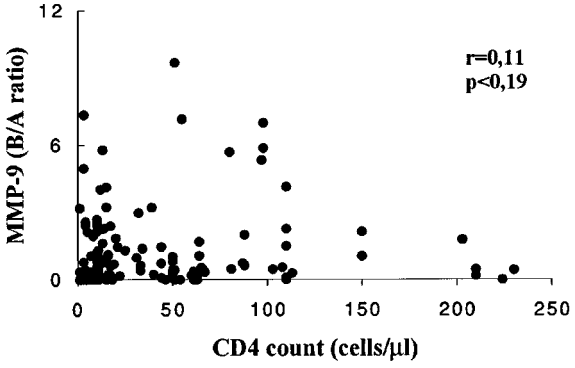

Figure 2 Relationship between CSF MMP-9 and CD4$^+$ T-cell count in the group of all HIV-infected patients. MMP-2 and MMP-9 activities were detected by zymography, quantitated as OD × mm^2 by scanning densitometry and computerised analysis and expressed as gelatinase B/A ratio. The levels of CSF MMP-9 did not correlate with CD4$^+$ T-cell count in the group of HIV-infected patients (Spearman rank test).

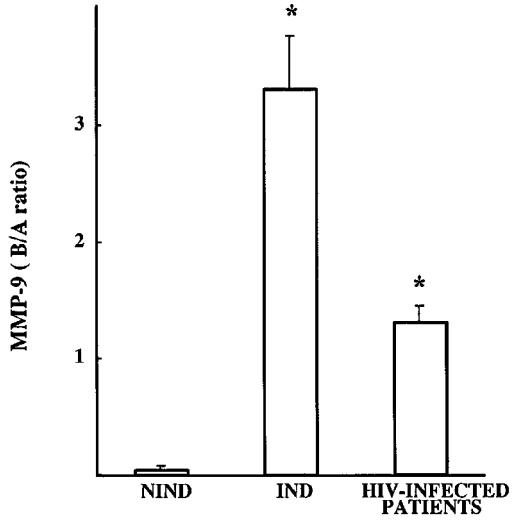

Figure 3 MMP-9 levels in CSF of HIV-seronegative (NIND, IND) and HIV-seropositive patients. MMP-9 levels are expressed as B/A ratio. Histograms represent the mean values of MMP-9 in the different patient groups. Bars indicate s.e.m. A significant difference between the groups was observed (one-way ANOVA, $F=25.02$, $P<0.001$). Asterisks indicate a significant difference *versus* the control group NIND. (Dunnett's test, $P<0.05$).

Relationship between MMP-9 activity and CSF cytosis

The number of CSF WBC of IND and HIV-infected patients with CMV-E and TBM were found to be significantly higher than in the CSF of NIND patients (Figure 5). Differential WBC counts showed that more than 90% of cells were neutrophils in the CSF of IND group and CMV-E, whereas in the other individual subgroups there was a predominance of mononuclear cells. The relationship between the MMP-9 activity in the CSF and the number of CSF WBC of each individual patient is shown in Figure 6A. A statistically significant correlation was found between the CSF cytosis and the MMP-9 in the pooled group of HIV$^+$ patients ($P<0.0001$; $r=0.45$). However, when the relationship between CSF MMP-9 activity and cytosis was calculated in every single subgroup of patients, a significant correlation was only observed for the group of ADC patients ($P<0.03$; $r=0.33$) (Figure 6B). No statistically significant correlation between MMP-9 in the CSF and cytosis was found in all the other stratified groups of HIV-infected patients (see Table 1 for statistical analysis data).

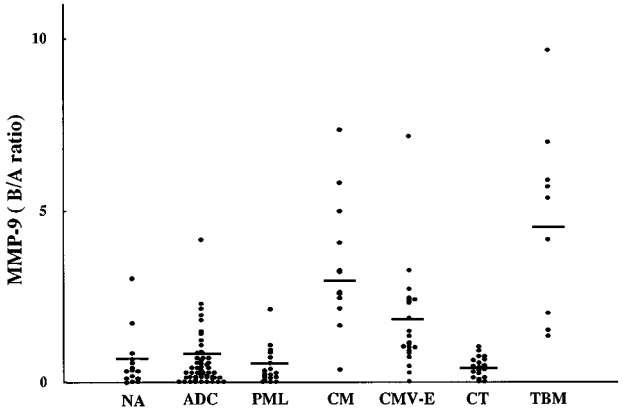

Figure 4 Distribution of MMP-9 levels (calculated as B/A ratio) in the CSF of 138 HIV-infected patients classified according to diagnosis. Groups of patients are: neurologically asymptomatic (NA); AIDS dementia complex (ADC); progressive multifocal leukoencephalopathy (PML); cryptococcal meningitis (CM); cytomegalovirus encephalitis (CMV-E); cerebral toxoplasmosis (CT); tuberculous meningitis (TBM). Lines indicate the mean values of each group. Significant differences between the groups were observed (one-way ANOVA, $F=26.22$, $P<0.001$). Significant differences in MMP-9 levels of CM, CMV-E and TBM groups *versus* the control group NA were observed. (Dunnett's *t*-test, $P<0.05$).

Discussion

Matrix metalloproteinases (MMPs), the 72 kDa gelatinase A (MMP-2) and 92 kDa gelatinase B (MMP-9), have recently been identified as mediators of brain injury in both demyelinating and infectious neurological disorders (Paul *et al*, 1998; Opdenakker and Van Damme, 1994) and several arguments suggest that they may be involved in the pathophysiology of HIV-infection of the brain (Weeks *et al*, 1993; Weeks, 1998). In this regard, it has been found that MMP activities can be up-regulated by a variety of mediators present in the CSF of HIV-infected patients such as cytokines and nitric oxide (Genis *et al*, 1992; Adamson *et al*, 1996) and increased MMP-9 secretion has been reported in the CNS infections with retrovirus human T-lymphotropic virus type I (HTLV-I) (Giraudon *et al*, 1996). Furthermore, it has been reported that human monocytes infected *in vitro* with HIV or stimulated with HIV Tat protein secrete MMPs capable of degrading basement membrane matrices thus facilitating extravasation of infected cells and development of HIV-associated neurologic damage and opportunistic infections in the brain (Dhawan *et al*, 1992, 1995; Lafrenie *et al*, 1996).

In the present study, we observed in CSF samples analysed by zymography the presence of both MMP-2 and MMP-9. MMP-2 was present in all CSF samples from HIV-seropositive and HIV-seronegative individuals, including those of subjects with NIND. This corroborates the findings from *in vitro* and *in vivo* studies that MMP-2 is a constitutive enzyme, which may be important in the normal turnover of the extracellular matrix during matrix remodelling (Paemen *et al*, 1994; Masure *et al*, 1990; Opdenakker and van Damme, 1994).

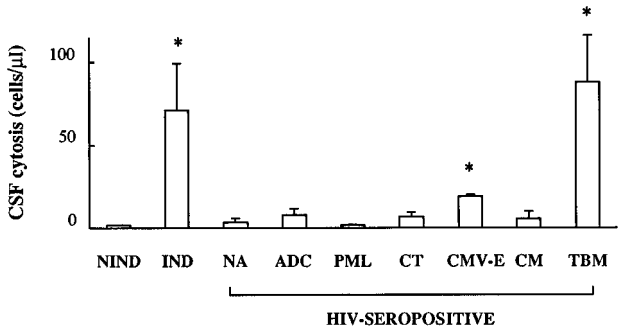

Figure 5 CSF cytosis (cells/μl) (mean $\pm$ s.e.m.) in HIV-seronegative groups (NIND and IND) and in HIV-seropositive groups, classified according to diagnosis. Significant differences between the groups were observed (one-way ANOVA, F=21.72, $P<0.001$). Asterisks indicate a significant difference *versus* the NIND control group (Dunnett's t-test, $P<0.05$). NIND=noninflammatory neurological diseases; IND=inflammatory neurologic diseases; NA=neurologically asymptomatic; ADC=AIDS dementia complex; PML=progressive multifocal leukoencephalopathy; CT=cerebral toxoplasmosis; CMV-E=cytomegalovirus encephalitis; CM=cryptococcal meningitis; TBM=tuberculous meningitis.

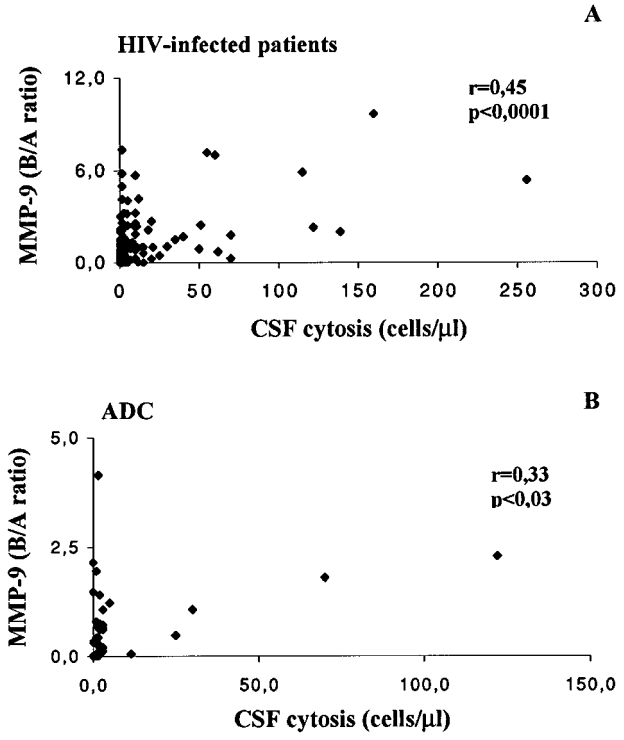

Figure 6 Relationship between CSF MMP-9 levels (calculated as B/A ratio) and CSF cytosis in the group of all HIV-infected patients (**A**) and in the subgroup of patients with AIDS dementia complex (ADC) (**B**). CSF cytosis correlated with MMP-9 levels in the group of HIV-infected patients and in the subgroup of ADC patients (Spearman rank test).

Table 1. Spearman correlation analysis between CSF cytosis and MMP-9.

Groups	r *Speraman*	P *value*
Total HIV infected patients	0.45	0.0001*
NA	0.37	0.18
ADC	0.33	0.03*
PML	−0.45	0.07
CM	−0.42	0.15
CMV	0.11	0.62
CT	0.28	0.22
TBM	0.47	0.20

Asterisks indicate statistically significant values.

As a contrast, MMP-9 was absent in the CSF of NIND controls, whereas the activity of this MMP was found in the 77–100% of CSF samples from HIV-infected patients, including those with ADC, CNS opportunistic infections or neurologically asymptomatic subjects. The lack of correlation between $CD4^+$ cells and MMP-9 levels indicates that the absence of MMP-9 in some HIV-infected individuals was not related to a more severe level of immunosuppression.

Sporer *et al* (1998) showed that CSF MMP-9 activity was present in 40% of HIV-infected patients and was significantly more frequent in patients with neurological complications. However, they did not find differences in the frequency of increased CSF MMP-9 activity between patients with or without CNS opportunistic infections. In the present study, we quantitatively analysed MMP-9 activity and the highest levels of this gelatinase were detected in the CSF of patients with CM, CMV-E and TBM and were comparable with those found in the CSF of patients with inflammatory neurological disorders such as MS or viral and bacterial meningitis (IND group). It is conceivable that the increased expression of MMP-9 activity in the CSF can be ascribed to the inflammatory state and the activation of the immune system during CNS opportunistic infection. HIV-related opportunistic infections of the brain are characterised by a marked activation of the cell-mediated immune responses in the subarachnoid space and we have previously shown increased concentrations of soluble immune activation markers (serum β2-microglobulin and neopterin) in the CSF of HIV-infected patients with fungal and protozoal CNS opportunistic infections and, to a less extent, in those with ADC (Mastroianni *et al*, 1993). In addition, we found that both TNF-α and soluble TNF receptors were up-regulated in the CSF of HIV-associated cryptococcosis, thus indicating an increased TNF activity in this inflammatory process (Mastroianni *et al*, 1996).

Two minor bands with gelatinolytic activity at apparent molecular weight 117 kDa and 130 kDa were observed by zymography in some CSF samples. The 130 kDa band was found in most samples with elevated MMP-9 levels and, as already reported, could probably represent a covalent complex between MMP-9 and the neutrophil MMP-9-associated lipocain (NGAL) (Kjeldsen *et al*, 1993). The identity of the 117 kDa band remains unknown. We exclude that this band could represent the TIMP-bound MMP-9, found by other authors in the CSF of MS patients (Rosenberg *et al*, 1996), since in our experiments it was present in all CSF samples and did not react with an anti-gelatinase B antibody (data not shown).

In our study, we determined the number of white blood cells (WBC) in the CSF of HIV-infected patients and compared the value with MMP-9 levels. We found a positive correlation between the presence of MMP-9 in the CSF and the number of CSF WBC only in ADC patients. In the other stratified HIV-infected patient populations we were unable to find a statistically significant correlation between these two parameters. In addition there was no association between the increase in any specific cell type (neutrophils or mononuclear cells) and MMP-9 levels. These findings suggest that MMP-9 could have a different origin than CSF cells; in this respect, neural cells, endothelial cells and infiltrated monocytes/macrophages might be possible candidates. Transendothelial migration of activated HIV-infected monocytes through the BBB may represent an important step in the pathobiological events in ADC which culminate in the secretion of neurotoxins leading to neuronal dysfunction (Gendelman *et al*, 1997; Nottet *et al*, 1996). It has been suggested that immune-activated monocytes/macrophages may induce the expression of adhesion molecules on the brain microvascular endothelium that allow binding and immigration of virus-infected cells into the CNS (Sasseville *et al*, 1994). The increased secretion of extracellular matrix degrading MMPs may contribute to the damage and disruption of the BBB, facilitating the entry of activated HIV-infected monocytes into the brain and thereby affecting the development of ADC.

In patients with CM the number of leukocytes and the increase of MMP-9 was not related. Indeed, the number of CSF cells was very low but levels of MMP-9 were highly variable and comparable with those of TBM and IND patients, in which the numbers of cells were high. Patients with CM have classically low CSF leukocyte counts (Eng *et al*, 1986; Zuger *et al*, 1986). Nevertheless, despite minimal leukocytosis, CM is associated with increased CSF expression of several mediators of inflammation. In particular, interleukin (IL)-8, a chemokine that plays an important role in the neutrophil recruitment to the site of infection, is released in large amounts in the CSF of patients with HIV-associated cryptococcosis (Chaka *et al*, 1997). Thus, despite the low number of inflammatory cells, CM seems to be characterised by a vigorous inflammatory response as reflected by the findings of increased expression of both MMP-9 and cytokines in the CSF compartment.

Increased levels of MMP-9 were also observed in patients with CMV-E. Several pathological studies have demonstrated that CMV can infect all cells in the brain, including astrocytes, neurons, oligodendroglia, cells of monocyte/macrophage lineage and capillary endothelial cells (Vinters *et al*, 1989). In CMV-E it is likely that CSF MMP-9 has its origin in endothelial cells; indeed the infection of brain capillary endothelial appears to be the site of entry of CMV into the CNS, and the virus then spreads from the choroid plexus into the CSF.

In conclusion, the data reported in this study indicate that the activity of MMP-9 was significantly increased in the CSF of HIV-infected patients, especially in those with CNS opportunistic infections. It is suggested that the overproduction of MMP-9 may contribute to an exaggerated inflammatory response promoting disease in the host. Further studies are needed to investigate whether the increased MMP-9 activity could lead to propagation of HIV infection within the brain and whether MMP-9 levels and MMP-9-related CNS damage decrease in patients under combined antiretroviral therapy. Conversely, it may also be interesting to study whether specific MMP inhibition alters HIV-associated neuropathology.

Materials and methods

Patients

One-hundred and thirty-eight HIV-seropositive individuals were recruited from the Department of Infectious and Tropical Diseases of the University 'La Sapienza', Rome between 1990 and 1997. The age ranged from 25 to 44 years; the median $CD4^+$ T-cell count was 15/μl (range, 1–230). The patients were grouped as follows: 40 patients with ADC; 17 patients with PML; 22 patients with cerebral toxoplasmosis (CT); 14 patients with cryptococcal meningitis (CM); 21 patients with cytomegalovirus (CMV) encephalitis (CMV-E); 9 patients with tuberculous meningitis (TBM); 15 neurologically asymptomatic subjects (NA). ADC was diagnosed according to the criteria recommended by the American Academy of Neurology (The Dana Consortium of Therapy, 1996). The diagnosis of CNS opportunistic infections was made on the basis of the clinical status, neurologic examination, compatible neuroimaging findings, routine studies of CSF, and detection of viral genomes by polymerase chain reaction (PCR). In the NA patients the clinical suspect of CNS involvement was not confirmed by neuroradiological assessment and CSF analysis.

Thirty-eight HIV-seronegative patients with inflammatory neurologic diseases (IND) (n=26) and noninflammatory neurological diseases (NIND) (n=12), ranging in age from 4 to 51 years, were selected as controls. Among the IND patients, one had aseptic meningitis and 12 had bacterial meningitis. The causative pathogens of bacterial meningitis were: *Neisseria meningitidis* (one), *Streptococcus pneumoniae* (one), *Haemophilus influenzae* type b (one), *Mycobacterium tuberculosis* (eight); in one purulent meningitis no organism was identified. Finally, 13 HIV-seronegative patients were affected by multiple sclerosis (MS). Among the 12 NIND patients with normal CSF findings, three were diagnosed with tension headache, one with hydrocephalus, eight with amyotrophic lateral sclerosis (ALS).

The design of the study was approved by the scientific committee of the Istituto Superiore di Sanità, Rome, Italy.

Handling of CSF samples

A total of 176 CSF samples were obtained by lumbar puncture and analysed for protein, glucose, cytology, and bacterial and fungal culture by using routine methods. Number of CSF white blood cells (WBC) and differential WBC counts were determined by standard methods from noncentrifuged samples. CSF samples were centrifuged at $300 \times g$, and the supernatants were stored at -70°C until used. The lumbar puncture in all patients was made as part of routine diagnostic protocols. A fully informed consent for the study was obtained from the patients.

Detection of gelatinase activity

The detection of gelatinase activity was performed by SDS–PAGE zymography according to a modification of the method of Heussen and Dowdle, (1980) as described by Masure *et al* (1990).

Briefly, 50 μl of each CSF sample were precipitated for 1 h at -20°C in cold aceton, then the samples were centrifuged and the pellets solubilised in 10 μl of loading buffer containing SDS. Samples were then applied on 10% polyacrylamide gels (10×10 cm) which had been copolymerised with 0.1% (w/v) gelatin. Stacking gels contained 5.4% polyacrylamide. Electrophoresis was carried out at 4°C for approximately 2 h at 100 V. After electrophoresis the gels were washed for 2×30 min in 2.5% (w/v) Triton X-100 in 100 mM Tris-HCl, pH 7.4 (washing buffer) in order to remove SDS and reactivate the enzyme, then incubated for 24 h at room temperature (R/T) in 100 mM Tris-HCl, pH 7.4 (developing buffer).

For the development of the enzyme activity, the substrate in the gels was stained with Coomassie brilliant blue R-250 and destained in methanol/acetic acid/H_2O. Gelatinase activity was detected as a white band on a blue background and was quantified by computerised image analysis through two-dimensional scanning densitometry. No variation was observed for gelatinase A which is constitutively expressed in body fluids and used as an internal control of sample processing (Masure *et al*, 1990). In this respect, to compare results taken from different gels and quantify gelatinase B (MMP-9), the ratio of gelatinase B to gelatinase A (B/A) was calculated for every sample (Gijbels *et al*, 1992; Paemen *et al*, 1994; Paul *et al*, 1998).

Statistical analysis

The existence of significant differences in B/A ratio or CSF cytosis in the different patient

groups was evaluated, after logarithmic transformation, by one way analysis of variance (ANOVA) followed by the *post hoc* comparison with the Dunnett's test. The correlation between MMP-9 and CSF cytosis and MMP-9 and $CD4^+$ T-cell count was performed by the Spearman rank test. Data were analysed by the statistical software SAS.

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