Journal of NeuroVirology (2000) 6, 187–201

www.jneurovirol.com

Relationship between viral load in blood, cerebrospinal fluid, brain tissue and isolated microglia with neurological disease in macaques infected with different strains of SIV

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The role of the viral burden in the brain for the pathogenesis of human immunodeficiency virus-associated neurological disorders is still unclear. To address this issue, we have quantified the viral load in plasma, cerebrospinal fluid (CSF) and brain tissue of macaques infected with simian immunodeficiency virus (SIV). We discovered that the viral strain used for infection determines the replicative capacity in microglial cells as well as the extent of neuropathological lesions and the occurrence of neurological symptoms. Moreover, the viral load in the brain parenchyma correlated with the development of overt neurological disease whereas the one in plasma did not. By comparing the viral load in three different compartments, we demonstrated that the viral burden in the CSF is influenced both by the viral replication in the periphery as well as in the brain parenchyma. According to these results, it is not the absolute amount of viral load in the CSF but rather the viral antigen contributed by the viral production within the brain which correlates with the development of neurological disease. In longitudinal studies, we observed that this autochthonous virus production, as evidenced by a ratio of the viral load in CSF to the one in plasma, takes place for a prolonged period of time before overt neurological signs are manifested. This finding suggests that this ratio could be used as a prognostic marker for immunodeficiency virus-induced neurological disease. *Journal of NeuroVirology* (2000) **6,** 187–201.

Keywords: central nervous system; encephalitis; brain pathology; T-cell; RT–PCR; *in situ* hybridization

Introduction

HIV infection not only leads to immunodeficiency but also invades the central nervous system (CNS) early in the course of infection. Whereas this persistent infection of the brain remains without neurological disease in the majority of asymptomatic patients, 20–30% of AIDS patients develop HIV-associated neurological disorders (McArthur *et al*, 1993). This syndrome, termed AIDS dementia complex (ADC) (Price *et al*, 1988) or HIV-cognitive-motor complex (Janssen *et al*, 1991), is characterised by a range of cognitive, motor and behavioural changes. Although productive infection of the CNS, which is found mainly in cells of monocytic/microglial origin (Budka *et al*, 1991; Masliah *et al*, 1994), is clearly a prerequisite for the development of ADC, the role of the viral replication within the CNS for the pathogenesis of this disorder has not yet been demonstrated conclusively. Using different methods to detect viral replication *in situ*, several studies have shown increased intrathecal viral replication in patients with ADC (Achim *et al*, 1994; Bell *et al*, 1996; Brew *et al*, 1995; Lazarini *et al*, 1997; Wiley *et al*, 1998). However, some of the patients in these studies displayed high levels of viral replication within the

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Received 1 September 1999; revised 13 December 1999; accepted 21 February 2000

CNS in the absence of neurological signs and other studies have failed to demonstrate a strong relationship between presence of viral antigen and RNA in brain tissue with neurological impairment (Glass *et al*, 1995; Johnson *et al*, 1996). Thus, additional host and viral factors may influence the development of ADC.

Since these studies have been performed on autopsy material, few data are available before the onset of AIDS symptoms (reviewed by Gray *et al*, 1996). Therefore, several groups have tried to evaluate the viral load in the CSF, expressed as viral antigen or RNA levels, as a diagnostic and prognostic window to CNS infection and development of neurological signs in cross-sectional studies (Bossi *et al*, 1998; Brew *et al*, 1997; Cinque *et al*, 1998; Conrad *et al*, 1995; DiStefano *et al*, 1997, 1998; Ellis *et al*, 1997; Gisslén *et al*, 1998; Martin *et al*, 1998; McArthur *et al*, 1997; Morris *et al*, 1998; Pratt *et al*, 1996; Robertson *et al*, 1998; Royal *et al*, 1994; Sei *et al*, 1996; Singer *et al*, 1994). Although the majority of these studies reported increased CSF viral load in neurological impaired patients, there was either a considerable overlap between the groups or such a correlation was found only in patients with low CD4 counts (Ellis *et al*, 1997). Moreover, CSF viral load often correlated with the load in the periphery (Ellis *et al*, 1997; Singer *et al*, 1994) or with the number of infiltrating cells (Ellis *et al*, 1997; Martin *et al*, 1998; Morris *et al*, 1998), whereas the relationship between CSF- and brain tissue RNA levels is less documented and was only weakly interrelated in demented subjects (McArthur *et al*, 1997). Thus it is still uncertain whether the viral load in the CSF reflects the situation within the brain parenchyma and can be used as diagnostic marker for the development of ADC (Price and Staprans, 1997).

In order to study the contribution of the viral replication in the plasma, in the brain parenchyma or in infiltrating $CD4^+$ T-cells to the viral load in the CSF and to correlate these parameters with the development of neurological disease under defined experimental settings, we have used the infection of rhesus macaques with simian immunodeficiency viruses (SIV) as a model system.

This animal model not only induces an AIDS-like disease but also mirrors many of the neuropathological changes found in HIV-infected patients (Czub *et al*, 1996; Lackner *et al*, 1991a; Ringler *et al*, 1988). In addition, close examination of SIV infected macaques with behavioural and electrophysiological tests has shown evidence for early cognition and motor impairment (Marcario *et al*, 1999; Murray *et al*, 1992) as well as neurophysiological abnormalities (Prospero-Garcia *et al*, 1996; Raymond *et al*, 1999), thus representing the most suitable animal model (Persidsky *et al*, 1995; Zink *et al*, 1998). Moreover, after infection with the viral strain SIVmac251MPBMC, macaques with poor anti viral immune response and rapid disease progression develop clinical signs of neurological disease at high frequency (Sopper *et al*, 1998). Using animals with different disease progression and infected with SIV-strains of different tropism, we have compared the viral load in plasma, CSF and in brain tissue. Our results show that high viral production by microglia, as found after infection with a macrophage tropic viral strain, correlates with the occurrence of neurological disease. Since according to our findings, the level of viral antigen in the CSF is influenced by the viral production both in the lymphocytic compartment and in the brain, an increased ratio of the viral antigen in the CSF compared to blood represents a suitable diagnostic marker for immunodeficiency virus induced cognitive-motor complex.

Results

Neurological disease in monkeys infected with different viral strains

In order to determine the impact of the viral strain used for inoculation on the occurrence of overt neurological disease, 43 animals infected with the macrophage tropic viral strain SIV mac251MPBMC and 35 animals infected with the lymphotropic viral clone SIVmac239 or closely related T-cell tropic derivatives (Lang *et al*., 1993, 1997), were carefully monitored for neurological signs by experienced veterinarians. Since previous studies demonstrated that both clinical neurological symptoms (Sopper *et al*, 1998) as well as SIV-induced encephalitis (SIVE) (Baskin *et al*, 1992; Westmoreland *et al*, 1998; Dean *et al*, 1993) develop more frequently among animals with rapid disease progression (i.e. AIDS within 6 months of infection) than among slow progressors, we have subgrouped the animals according to the disease progression (Table 1). Similar to another cohort of animals (Westmoreland *et al*, 1998) the mean time until development of AIDS was not significantly different between rapid progressors infected with either SIVmac239 or SIVmac251MPBMC. However, an influence of the virus used for inoculation on the incidence of neurological symptoms was observed in the two groups of the rapid progressor group which reached statistical significance ($P \leqslant 0.05$). Whereas 50% of the rapid progressors infected with SIVmac251MPBMC developed one or more of the following neurological signs such as ataxia (n=5), seizure (n=2), opisthotonus (n=1) and apathy (n=1), recently described in more detail (Sopper *et al*, 1998), none of the rapid progressors infected with the viral clone SIVmac239 showed evidence of clinical neurological disease. Pathological examination revealed severe SIVE in the absence of opportunistic infections and tumours of the CNS in all SIVmac251MPBMC-infected rapid progressors (Figure 1a). In contrast, histological alterations such as inflammatory no-

Table 1 Incidence of clinical neurological signs and extent of SIVE in Rhesus monkeys infected with different strains of SIV

	SIVmac239			*SIVmac251 MPBMC*		
		AIDS			*AIDS*	
	Asymptomatic	*Slow*	*Rapid*	*Asymptomatic*	*Slow*	*Rapid*
N	15	8	12	24	7	12
w.p.i.	NA	4.05 ± 84	19.5 ± 6	NA	58.3 ± 23	18.1 ± 6
neuro	0	1 (12.5)	0	0	1 (14.3)	6 (50)*
SIVE score	ND	ND	3 ± 2.8	ND	ND	6.4 ± 1.4*

N, number of animals; w.p.i., weeks until development of AIDS (mean ± s.d.); NA, not applicable; neuro, number (%) of animals with clinical neurological signs. *, significantly different compared to SIVmac239 infected rapid progressors. SIVE score, is shown as mean ± s.d. of eight rapid progressors infected with the respective viruses. ND, not determined.

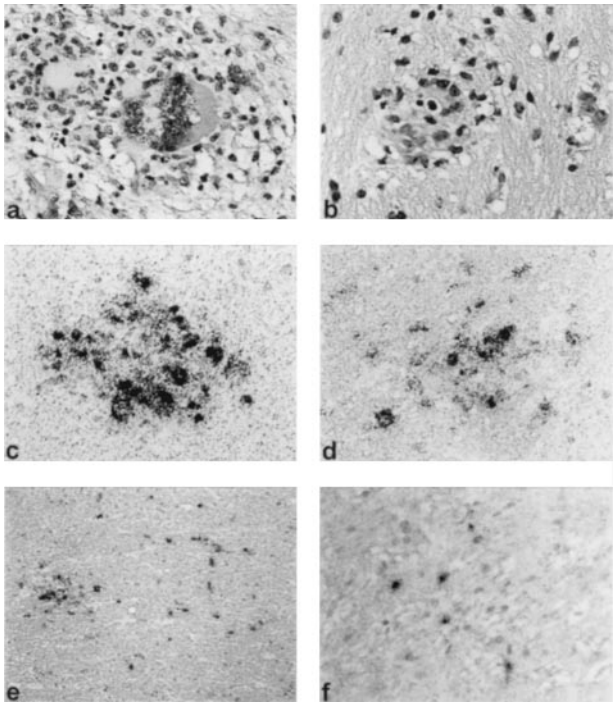

Figure 1 Neuropathological lesions (**a**, **b**) and viral RNA-expressing cells (**c**–**f**) in brains of rapid progressors infected with SIVmac251MPBMC (left panels, SIVE score 8) or SIVmac239 (right panels, SIVE score 3). (**a**) H&E stain, original magnification × 160; (**b**) H&E stain, original magnification × 250. (**c**, **d**, **f**) *in situ* hybridization, original magnification × 160; (**e**) *in situ* hybridization, original magnification × 100. Brain sections were hybridized with identical SIV-specific probes and processed in parallel.

dules and multinucleated giant cells, characteristic for SIVE, were found in a lower proportion of rapid progressors infected with SIVmac239 (75%). In addition, the severity of histological lesions was less pronounced (Figure 1b). This difference is further evidenced by a semiquantitative evaluation of the extent of SIVE in 16 rapid progressors infected with the two different viral strains (Table 1). The cumulative encephalitic score was significantly higher in rapid progressors infected with SIVmac251MPBMC (ranging from 4–8 in individual animals) compared to those infected with SIVmac239 (range 0–8). In addition, among animals infected with SIVmac251MPBMC those with neurological signs had a significantly higher SIVE score (7.5 ± 0.6) than those without neurological signs (5.3 ± 0.9). Thus, the extent of SIVE correlated with the viral inoculum and the occurrence of clinically overt neurological signs.

Correlation of neurological disease with viral burden in plasma, CSF and brain parenchyma at necropsy

In order to determine whether a difference in the overall viral replication was responsible for the different incidence of neurological disease, we have compared the antigenemia in plasma of monkeys inoculated with the two viral strains (Table 2, Figure 2a). Since among asymptomatic animals and slow progressors with AIDS only a few monkeys infected with each of the viruses displayed viral antigen levels above the detection limit of the assay, we have only discriminated between the viruses in the group of rapid progressors. In asymptomatic animals p27 could be detected only rarely. Among animals with AIDS, rapid progressors displayed the highest levels of p27 in plasma regardless of the virus used for infection. In addition, within the group of SIVmac251MPBMC-infected animals, no difference in the plasma viraemia could be found between neurological symptomatic and asymptomatic animals. Thus, the level of viral replication in the periphery does not correlate with the development of neurological disease. In contrast, p27 levels in CSF were higher in rapid progressors infected with SIVmac251MPBMC than in monkeys inoculated with SIVmac239 ($P \leqslant 0.05$) (Figure 2b). Moreover, animals with neurological signs had higher amounts of viral antigen in the CSF than animals infected with the same virus but without neurological signs. Thus, the p27 levels in CSF correlate with the strain of the virus used for infection and the occurrence of neurological disease in rapid progressors.

Table 2 Viral antigen level in plasma, CSF and microglia supernatants of SIV-infected macaques at necropsy

Animals number	*SIVmac strain inoculated*	*Clinical status (disease course)*	*Necropsy (w.p.i.)*	*p27 (pg/ml)*		
				Plasma	*CSF*	*Microglia*
1	251MPBMC	asymptomatic	1	374	14	<3
2	251MPBMC	asymptomatic	2	243	20	<3
3	251MPBMC	asymptomatic	12	ND	<3	23
4	251MPBMC	asymptomatic	16	ND	<3	8
5	239	asymptomatic	21	591	4	16
6	239	asymptomatic	22	ND	<3	9
7	251MPBMC	asymptomatic	33	585	<3	<3
8	239	AIDS (rapid)	15	100 000	2310	626
9	239	AIDS (rapid)	19	3350	<3	<3
10	239	AIDS (rapid)	20	ND	ND	140
11	239	AIDS (rapid)	20	200 000	4650	97
12	239	AIDS (rapid)	20	ND	ND	5000
13	239	AIDS (rapid)	20	2590	126	ND
14	239	AIDS (rapid)	20	1670	23	6
15	239	AIDS (rapid)	28	1590	10	31
16	239	AIDS (slow)	33	7	ND	<3
17	239	AIDS (slow)	44	820	ND	<3
18	239	AIDS (slow)	126	46	<3	ND
19	251MPBMC	AIDS (rapid)	12	23 000	12 430	1613
20	251MPBMC	AIDS (rapid)	13	51 000	17 000	10 800
21	251MPBMC	AIDS (rapid)	13	6330	3890	ND
22	251MPBMC	AIDS (rapid)	14	71 160	8270	1960
23	251MPBMC	AIDS (rapid)	15	5890	5770	28 230
24	251MPBMC	AIDS (rapid)	16	34 780	1520	4000
25	251MPBMC	AIDS (rapid)	26	28 800	25 400	4850
26	251MPBMC	AIDS (rapid)	28	7990	1460	ND
27	251MPBMC	AIDS (slow)	35	146	<3	<3
28	251MPBMC	AIDS (slow)	55	16	<3	ND
29	251MPBMC	AIDS (slow)	57	123	<3	<3
30	251MPBMC	AIDS (slow)	94	110	<3	ND

Viral antigen levels were determined by ELISA. Only those animals of which viral antigen levels in at least one compartment were above the detection limit of the assay are listed. ND, not determined.

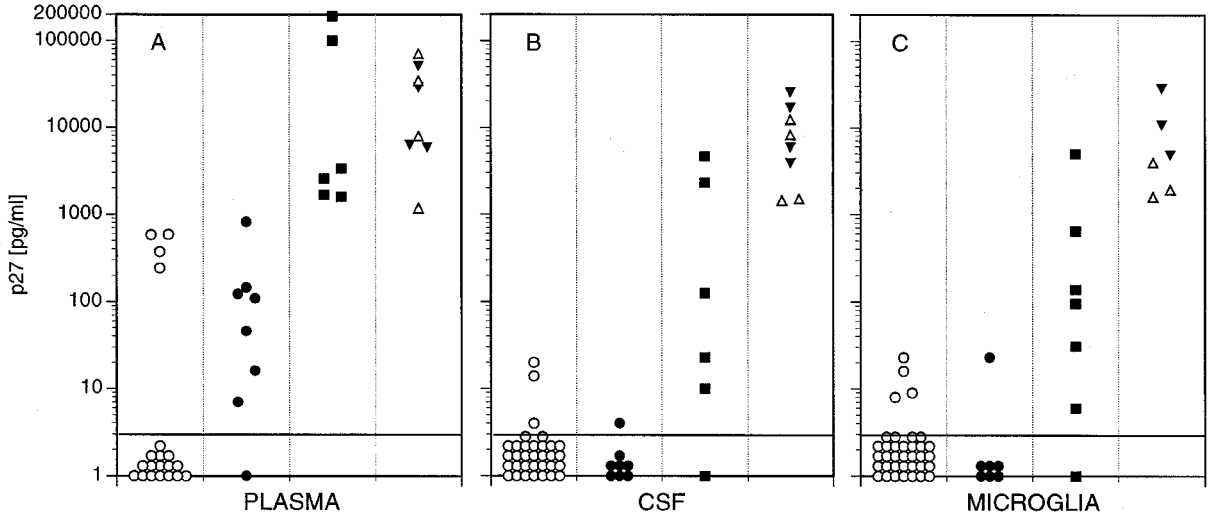

Figure 2 Viral antigen in plasma (**A**), CSF (**B**) and produced by isolated microglia (**C**) of SIV-infected Rhesus monkeys at necropsy. p27 concentrations were determined by ELISA in samples from asymptomatic animals sacrified at predetermined time points (○), slow progressors with AIDS (●), rapid progressors infected with SIV239 (■) and rapid progressors infected with SIV251MPBMC with (▼) or without clinical neurological disease (△).

In order to quantify the overall viral production in the brain parenchyma, we have isolated haematopoetic mononuclear cells from the brain parenchyma using a Percoll gradient technique (Sedgwick *et al*, 1991) and have analysed these cells for p27 production and infectious virus. Cells in this

preparation were >90% microglial cells with usually less than 3% of infiltrating macrophages as determined by flow cytometry (data not shown). These cells were cultured for only 20 h in order to reflect the situation *in vivo* as closely as possible. Among supernatants of microglial cells isolated from asymptomatic animals, only few cases (four out of 36) with low production of p27 were detected (Figure 2c). Slow progressors with AIDS showed comparable levels of the core antigen. In contrast, all but one culture from rapid progressors contained viral antigen. The amount of p27 however, was statistically higher in supernatants of animals infected with SIVmac251MPBMC compared to rapid progressors infected with the viral clone SIVmac239 ($P \leqslant 0.01$). The highest levels of viral antigen was found in cultures from animals with overt neurological signs, reaching statistical significance ($P \leqslant 0.05$) when compared with rapid progressors infected with the same virus but without neurological signs. In addition, microglia isolated from these animals produced infectious virus titers up to 1×10^4 (data not shown). Thus, the viral antigen levels in microglia supernatants correlated well with the development of neurological signs.

Since lymphocytes comigrate with microglial cells in the gradient used for isolation, we were concerned about a possible contribution of $CD4^+$ T-cells to the production of viral antigen in these short term cultures. We have therefore determined the percentage of $CD4^+$ T-cells among the isolated cells by flow cytometry and compared this with p27 levels in the supernatant (Figure 3). In most preparations, the proportion of $CD4^+$ T-cells was below 1%. In addition, a negative correlation between the percentage of this cell population and the amount of viral antigen in p27 positive cultures was observed. For selected animals, we studied viral antigen production of lymph node cells under the same conditions. The $CD4^+$ T-cells present in these cultures ($\sim$20%) did not yield sufficiently high viral antigen levels to account for the amount of p27 present in microglia supernatants (data not shown). In addition, no correlation could be found between viral antigen levels in LN- and microglia cultures. Thus, these cultures truly reflect the production of viral antigen by microglial cells or infiltrating macrophages. However, some asymptomatic animals with relatively high proportions of $CD4^+$ T-cells showed measurable levels of p27. This suggests that $CD4^+$ T-cells may contribute to the brain parenchymal viral replication in the early stage of the disease.

As an additional measure of the viral replication in the brain parenchyma we have determined the viral RNA expression by *in situ* hybridization (Figure 1c–f). Positive signals were abundant in multinucleated giant cells and inflammatory nodules (Figure 1c, d) but could also be found

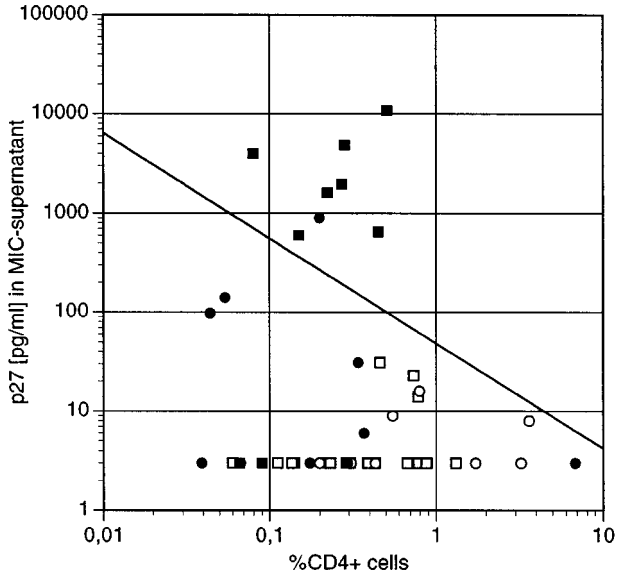

Figure 3 Relationship between viral antigen in supernatant of isolated brain cells and proportion of $CD4^+$ T-cells among isolated brain cells from asymptomatic animals sacrified at predetermined time points infected with SIVmac251MPBMC (□) or SIVmac239 (○) and animals with AIDS infected with either SIVmac251MPBMC (■) or SIVmac239 (●).

in parenchymal microglia cells especially of SIVmac251MPBMC-infected rapid progressors (Figure 1e). Semiquantitative assessment of *in situ* hybridization signals revealed between 1 and 10 viral RNA expressing cells per cm^2 in neocortical brain regions of asymptomatic animals and slow progressors with AIDS (Figure 4a). This number was not increased substantially in rapid progressors infected with SIV239. In contrast, rapid progressors infected with SIVmac251MPBMC revealed on average 220 viral RNA expressing cells/cm^2, which was significantly different to the other groups ($P \leqslant 0.05$ and $P \leqslant 0.01$). In addition, the number of productively infected cells correlated with the SIVE score (r^2=0.8). Whereas RNA expressing cells were found in every SIV-infected animal, quantification of viral RNA in brain tissue by a highly sensitive QC–RT–PCR was possible only in 54% of the asymptomatic monkeys (Figure 4b). In contrast, in the brains of SIVmac251MPBMC-infected macaques with neurological signs the viral RNA levels reached 8×10^7 RNA equivalents/g brain tissue. Thus, the dynamic range of this method was similar to the one of the detection of viral antigen in microglia supernatants but higher than the one of *in situ* hybridization.

In order to define the relative contribution of the peripheral and the brain parenchymal viral replication to the viral burden in the CSF, we have compared the viral antigen levels in CSF

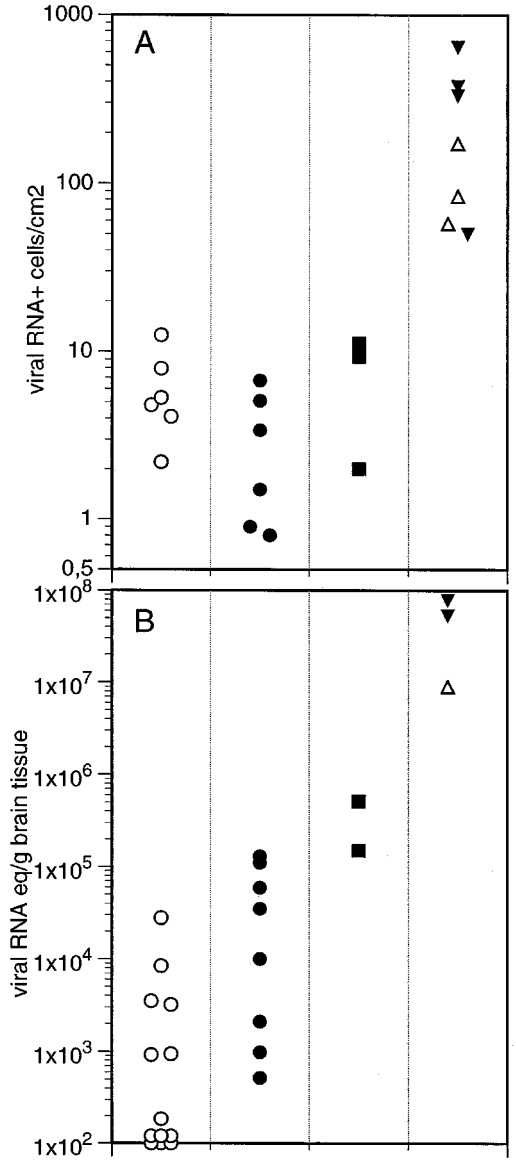

Figure 4 Numbers of viral RNA expressing cells and viral RNA equivalents in the CNS of SIV-infected Rhesus monkeys. Brain tissue from asymptomatic animals sacrified at predetermined time points (○), slow progressors with AIDS (●), rapid progressors infected with SIVmac239 (■) and rapid progressors infected with SIVmac251MPBMC with (▼) or without (△) clinical neurological disease was analysed for expression of viral RNA by *in situ* hybridization (**A**) and QC–RT–PCR (**B**). Brain sections of asymptomatic animals were hybridized with a cocktail of env, nef and gag probes with increased sensitivity compared to the gag specific probe used for the other groups of animals.

with the one in microglia cultures and plasma at necropsy of animals grouped according to the virus used for infection. In both groups of animals, the viral antigen levels in CSF correlated with the amount of p27 in microglial cultures (Figure 5a, r^2=0.83 and 0.71 respectively). Similarly, the concentration of p27 in CSF increases in both groups in good correlation with the rising viral antigen levels in the periphery (Figure 5b, r^2=0.86 and 0.86 respectively). However, animals infected with SIVmac239 displayed a higher ratio of p27 in CSF to p27 in microglia supernatants than animals infected with SIVmac251MPBMC (Figure 5c) suggesting an additional source of the viral antigen in CSF. In contrast, over the complete range of concentrations, the amount of p27 in CSF in relation to the one in plasma is higher in animals infected with SIVmac251MPBMC, resulting in a higher ratio of $p27_{CSF}/p27_{plasma}$ (Figure 5d). Whereas the CSF p27 levels of animals infected with SIVmac239 were on average only 4% of plasma p27 levels, the mean levels in the CSF of monkeys infected with SIVmac251MPBMC reached about one third of the plasma levels. This difference of the ratios $p27_{CSF}/p27_{plasma}$ in the two groups of animals was statistically significant ($P \leqslant 0.01$), Thus, viral antigen found in CSF after infection with SIVmac251MPBMC is in most part the result of an autochthonous production within the brain, whereas the levels in SIV239-infected animals rather depend upon the viral replication in the periphery. In addition, within the group of rapid progressors infected with SIVmac251MPBMC, monkeys with neurological disease had a statistically higher ($P \leqslant 0.01$) $p27_{CSF}/p27_{plasma}$ ratio than animals without neurological signs. Therefore this ratio could be used as a diagnostic marker for SIV-induced neurological disease.

Kinetics of viral antigen and CD4$^+$ T-cells in CSF

In the present study we have revealed a strong correlation of the viral replication in the brain tissue with the phenotype of the virus used for inoculation and the incidence of neurological signs. However, not all of the rapid progressors infected with SIVmac251MPBMC developed neurological signs and some monkeys displayed high CSF viral load in the absence of overt clinical symptoms of neurological disease. In addition, viral replication within microglial cells can not be used as prognostic marker for immunodeficiency virus-induced neurological disease. Therefore, we sought to determine the prognostic potential of the viral load in the CSF in longitudinal studies.

In order to exclude an influence of iatrogenic blood contamination or leakage of the BBB on viral antigen levels we have determined the quotient of the albumin concentrations in plasma and CSF (Q_{Alb}). None of the animals tested showed gross disruptions of the BBB as judged by an increase in the Q_{Alb} higher than twice the standard deviation of the individual fluctuations before infection (data not shown). Only one slow progressor displayed a transient increase in the albumin quotient at 2 w.p.i. Concomitantly, this

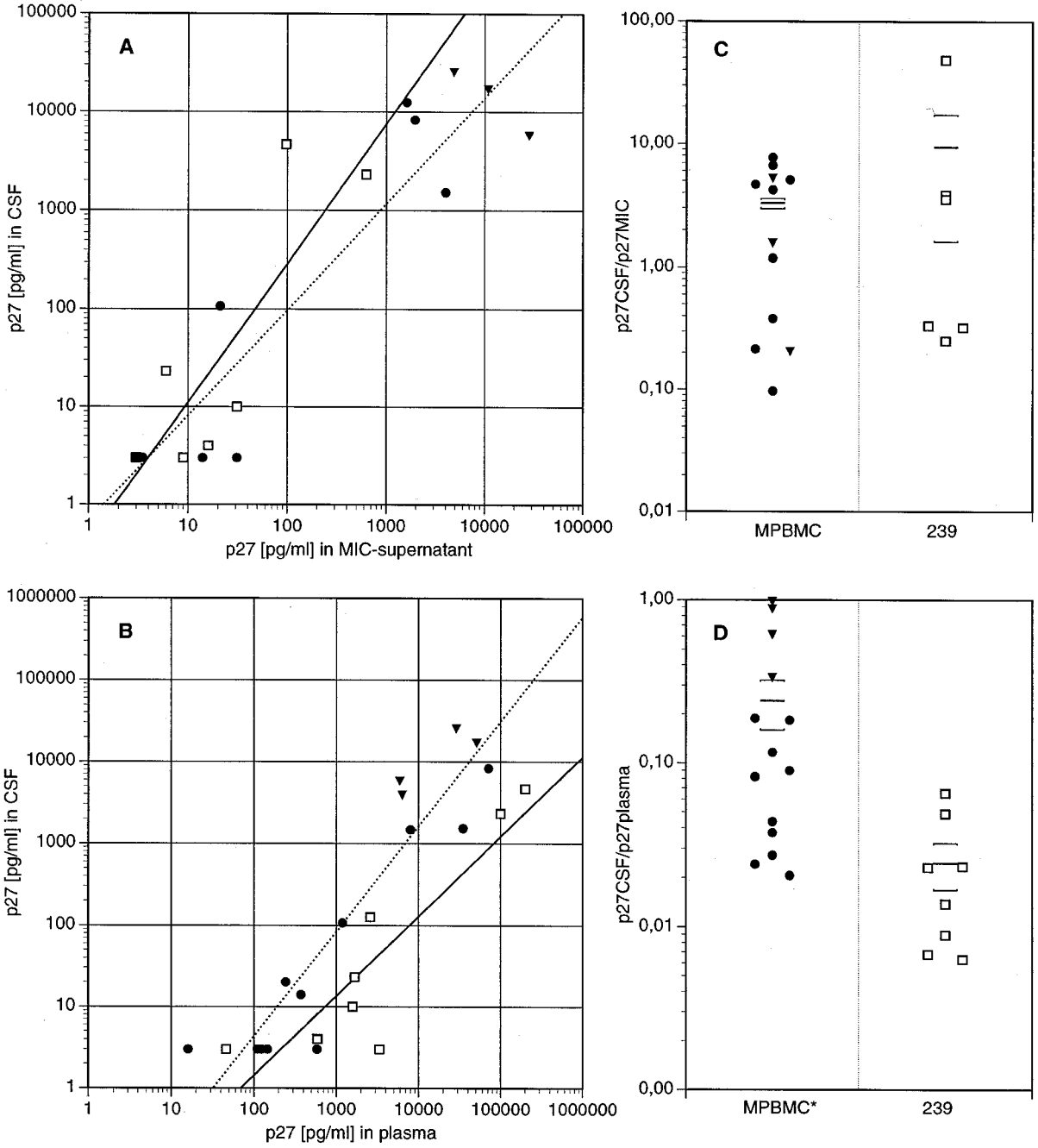

Figure 5 Relationship between viral antigen levels in paired samples of CSF and microglia supernatants (**A**, **C**) or of CSF and plasma (**B**, **D**) at necropsy of SIVmac239- (□) and SIVmac251MPBMC-infected monkeys with (▼) and without (●) clinical neurological signs. Linear regression in (**A**) and (**C**) revealed significant correlations between viral antigen levels in the different compartments (Spearman's rank $P \leqslant 0.05$) for SIVmac239- (full line) and SIVmac251MPBMC-infected animals (dotted line). In (**C**) and (**D**), the mean ratios $\pm$ s.e.m. of p27 of each group are depicted as horizontal bar.* The ratio $p27_{CSF}/p27_{plasma}$ was significantly higher in SIVmac251MPBMC- than in SIV239-infected animals ($P \leqslant 0.05$).

animal also showed the highest numbers of infiltrating $CD4^+$ T-cells in the CSF among all animals studied so far (Figure 6d).

In parallel with the initial plasma viraemia, at 2–3 w.p.i. viral antigen was also found in cell-free CSF of all animals investigated (Figure 6a, b). At this time point, numbers of $CD4^+$ T-cells were increased in the CSF of some slow progressors (Figure 6d) and infiltrating, viral RNA expressing lymphocytes could be found in the meninges and

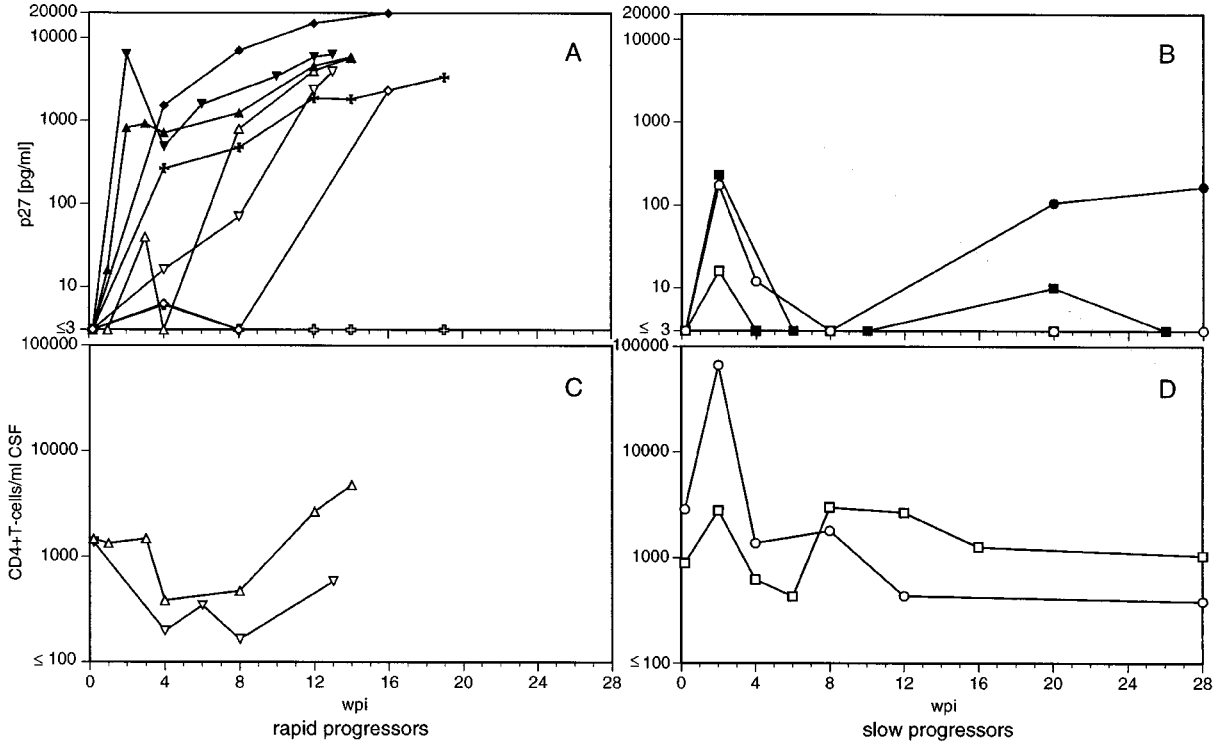

Figure 6 Kinetics of viral antigen (**A**, **B**) in paired samples of plasma (closed symbols) and CSF (open symbols) and $CD4^+$ T-cell numbers (**C**, **D**) in CSF. Values of SIV-infected slow progressors (**B**, **D**) until 28 w.p.i. and rapid progressors (**A**, **C**) infected with either SIVmac239 (◆, ◇, ✚, ✙) or SIVmac251MPBMC (▼, ▽, ▲, △) until euthanasia are shown.

perivascular space of brain vessels, whereas only a few microglia cells positive for viral RNA were detected. Later in the course of the disease, the kinetics of viral antigen levels in plasma and CSF differed between rapid and slow progressors as a result of the different virus-specific immune responses in these two groups (Sopper *et al*, 1998). Whereas the strong immune response of slow progressors was able to restrict viral replication to low or undetectable levels in plasma and in CSF, the amount of p27 found in plasma and CSF of rapid progressors continued to rise after a short decline at 4 w.p.i. The production of viral antigen in the CSF of rapid progressors infected with SIVmac251MPBMC and exhibiting neurological signs was not accompanied by an infiltration of $CD4^+$ T-cells and increased more steeply than the one in the periphery, reaching almost plasma levels at the time of death. Kinetic data from rapid progressors infected with SIVmac239 indicate comparable levels of viral antigen in blood but much lower amounts in the CSF (Figure 6a).

This fact is even more accentuated when the ratio of $p27_{CSF}/p27_{plasma}$ was determined (Figure 7). In all rapid progressors infected with SIVmac251MPBMC, this ratio eventually rises above the mean plus twice the standard deviation of the values at necropsy from animals infected with SIV239. In addition among these animals, macaques with neurological disease displayed a higher p27 ratio for a prolonged period starting as early as 6 w.p.i. This suggests that not only the magnitude but

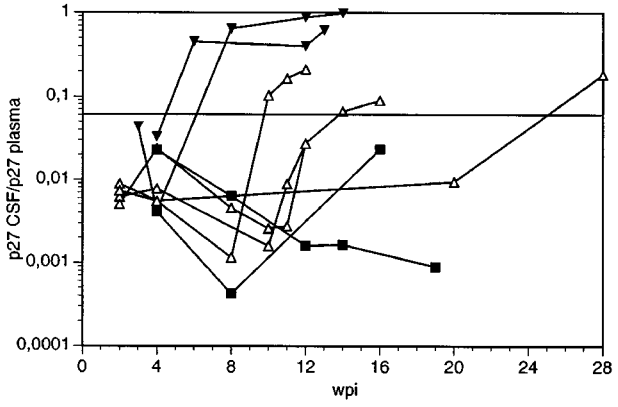

Figure 7 Kinetics of the ratio of viral antigen in paired samples of CSF and plasma of SIVmac239- (■) and SIVmac251MPBMC-infected rapid progressors with (▼) and without clinical neurological signs (△) until euthanasia. The line represents the mean ratio $p27_{CSF}/p27_{plasma}$ +2 s.d. of all SIVmac239-infected animals at necropsy.

also the duration of the intrathecal virus replication is responsible for the development of clinically overt neurological signs. Moreover, since in animals which later developed neurological disease the p27 ratio was higher than in neurological asymptomatic monkeys already 6–8 weeks before death, this parameter seems to be of prognostic value.

Discussion

Brain viral load and occurrence of neurological disease

In the present study we have used the infection of macaques with SIV to compare the occurrence of overt clinical signs of neurological disease and neuropathological lesions with the level of viral replication in the brain parenchyma as evidenced by ISH and QC–RT–PCR and the production of viral antigen and infectious virus by isolated microglial cells. Microglial cells are the main target for productive immunodeficiency virus infection within the CNS (Dickson *et al*, 1994; Takahashi *et al*, 1996). The comparison of the number of viral RNA expressing cells and viral RNA load in brain tissue with viral antigen in microglia supernatants demonstrated that the latter closely reflects the situation in the brain parenchyma. Similarly, in a study comparing the number of gp41 positive cells with the amount of viral antigen extracted from brain tissue, matching results were found with both methods (Achim *et al*, 1993). Although ISH is more sensitive and, as shown previously (Boche *et al*, 1999; Chakrabarti *et al*, 1991; Lackner *et al*, 1991b), can detect single positive cells in the asymptomatic phase when microglial cultures were negative for viral antigen, quantification of the global viral replication is easier to perform with isolated microglial cells. In addition, in animals with a high viral production the actual number of RNA expressing cells might have been underestimated due to confluent signals, which may have resulted in the lower dynamic range compared to our results obtained by QC–RT–PCR and isolated microglia cells. Other techniques such as measuring viral antigen or infectious virus in brain homogenates (Achim *et al*, 1993; Adamson *et al*, 1996; Sharma *et al*, 1992b) or viral RNA in the tissue (Lazarini *et al*, 1997; McArthur *et al*, 1997; Wiley *et al*, 1998) can only cover small regions of the brain which display different expression of virus (Wiley *et al*, 1998).

With these methods, we could demonstrate that high viral replication in microglial cells but not in the periphery is a prerequisite for the occurrence of neurological signs in SIV-infected monkeys. Similar to our results, several reports have found a good correlation between proviral copies and/or p24 immunostaining and both HIV-encephalitis and ADC (Bell *et al*, 1996; Brew *et al*, 1995; Wiley and Achim, 1994). In addition, in a very recent study a correlation between viral RNA levels in brain tissue and the severity of SIVE has been demonstrated in a limited number of SIV-infected macaques (Zink *et al*, 1999). However, this concordance is not absolute and other studies have detected a correlation of only borderline significance (Glass *et al*, 1995) or did not find increased viral replication in the brain tissue of demented patients (Johnson *et al*, 1996; McArthur *et al*, 1997). Similarly, although those three animals with clinical neurological signs, for which for viral production by microglia was determined, also had the highest viral antigen levels in microglia supernatants there were also some animals with high viral antigen levels without overt neurological disease. In these animals, but possibly also in animals with lower viral replication within the CNS, subtle deterioration of neurological functions as shown for small cohorts of SIV-infected animals with a battery of sophisticated methods (Marcario *et al*, 1999; Murray *et al*, 1992; Prospero-Garcia *et al*, 1996; Raymond *et al*, 1999) might have remained undetected due to our insensitive assessment. On the other hand, among the animals with low viral replication in microglia, we did not find neurological symptomatic animals, which suggests that high viral replication is necessary for development of overt neurological disease. One possible reason for the incomplete correlation between high viral load and neurological disease in these reports is that autopsy material represents only a snap shot of the viral replication at the time of death. Our kinetic studies on the viral load in the CSF suggest that increased intrathecal viral replication must occur for a prolonged period to induce severe neurological signs. Similarly, in the only longitudinal study with HIV-infected patients a significant increase of viral RNA in CSF but not in plasma was already found in the asymptomatic phase (Gisslén *et al*, 1998).

According to our results, the level of viral replication within the CNS and thus the occurrence of neurological disease is influenced by several host- and virus factors.

First, the higher incidence of neurological signs among rapid progressing animals, which do not mount an intrathecal virus-specific immune response suggests a protective role of the immune system on the outcome of the CNS-infection (Sopper *et al*, 1998). Animals with a strong intrathecal virus-specific immune response are able to restrict the viral replication. In these slow progressors, the viral replication was usually below the detection limit of our assays. Only late in the course of disease, concomitant with the deterioration of the immune system, low levels of viral replication were found in microglia isolated from slow progressors. If these animals were not euthanised but intensively treated for opportunistic infections and the wasting, increased viral replication for a prolonged period would have occurred,

possibly leading to overt neurological disease as in the two slow progressors with neurological signs. Unfortunately, no data were available about the viral replication in the CNS of these two animals.

Second, the occurrence of neurological signs and the extent of SIVE are dependent on the viral strain used for infection, whereas the survival time and the systemic symptoms were not different. Together with the fact that in some animals neurological signs developed before the onset of other AIDS defining diseases, this strongly suggests that these neurological signs are not the effects of a systemic illness. Similar to our results, previous reports have documented a higher incidence of SIV encephalitis after infection with the macrophage tropic virus SIVmac251 compared to infection with the lymphotropic viral clone SIVmac239 (Westmoreland *et al*, 1998) and a correlation of the presence of macrophage tropic viruses in the CSF with the occurrence of ADC in HIV-infected patients (Brew *et al*, 1996). However, as shown for our cohort of animals, macaques infected with the lymphotropic clone may as well develop SIVE (Desrosiers *et al*, 1991), but not clinical neurological disease (Sharma *et al*, 1992a). This fact can be explained by either a quantitative difference of SIVE as in the present study or a difference in the duration of these histopathological changes before death. In addition, the results of our study suggest that it is the increased replicative phenotype of neuropathogenic viruses in microglia rather than a special neurotoxic determinant which leads to the development of neurological disease.

Third, since only about 50% of the animals infected with the neurovirulent strain SIVmac251MPBMC and with a poor anti viral immune response developed neurological signs, additional factors may contribute to the pathogenesis of ADC. Animals with neurological signs displayed a higher intrathecal virus production for several weeks before necropsy compared to neurological inconspicuous monkeys infected with the same virus. Two mechanisms may be responsible for this difference. First, mutations leading to an increased replicative capacity in microglia such as shown for SIVmac239 (Kodama *et al*, 1993; Sharma *et al*, 1992b) may have occurred early in animals with neurological signs. On the other hand, since we have used outbred animals, additional host factors such as subtle differences in the virus specific immune response which remained undetected or a higher susceptibility of the microglia of the neurological symptomatic animals to SIV infection may also influence the viral replication and thus the occurrence of neurological disease. In this context, it is possible that the microglia of these animals were initially more activated. Immunodeficiency viruses replicate better in activated cells and infection of monocytes/microglia itself results in increased activation and secretion of cytokines. These cytokines could in turn activate surrounding uninfected cells (Brinkmann *et al*, 1993; Sopper *et al*, 1996), resulting in an autostimulatory cycle that could lead to high viral replication and strong activation of microglia. Activation of microglia has been documented in HIV-infected patients (Achim *et al*, 1991; McGeer *et al*, 1988; Weis *et al*, 1994) and SIV-infected macaques (Hurtrel *et al*, 1993) and seems to be correlated with ADC (Glass *et al*, 1995; Johnson *et al*, 1996). Whether neurotoxicity of viral proteins themselves or harmful metabolic products of the activated cells cause neuronal damage remains to be determined.

Relationship between amount of virus in brain tissue, CSF and periphery

High amounts of viral antigen were detected in CSF not only when viral production by microglial cells was very high, but also in some situations when viral RNA expressing cells could be detected only rarely in the brain parenchyma such as early after infection and in rapid progressors infected with lymphotropic virus. Since the BBB was intact in these animals, the viral antigen had to be produced intrathecally. One of the possible sources of the viral antigen in CSF at this time point are activated $CD4^+$ T-cells which represent the main reservoir for productive immunodeficiency virus replication and which are able to cross the BBB. The correlation between the number of infiltrating $CD4^+$ T-cells in the CSF and the presence of viral RNA expressing lymphocytes in the perivascular space in the first few weeks after infection suggests that the extravasating $CD4^+$ T-lymphocytes are responsible for the viral production within the CNS early after infection. This is in line with previous findings which demonstrated a correlation between the number of infiltrating cells and viral RNA in CSF (Ellis *et al*, 1997; Martin *et al*, 1998; Morris *et al*, 1998). According to these reports, the number of CSF-cells is influenced by the absolute $CD4^+$ T-cell counts in the periphery, reflecting the state of the immune system, and the occurrence of opportunistic infections within the CNS. The kinetic data of our cohort of animals in the absence of opportunistic infections suggest that the intrathecal SIV-specific immune response acts as an additional factor determining the number of infiltrating $CD4^+$ T-cells in the CSF. However, other cell populations such as endothelial cells and cells of the chorioid plexus may also have added to the viral load in the CSF. Later in the course of the disease, infected microglia contribute increasingly to the viral antigen in CSF, augmenting the ratio of p27 in CSF and plasma. In animals with neurological signs this ratio is higher for several weeks before death than in neurological asymptomatic animals, providing a prognostic tool for the development of neurological disease. In addition, CD4 cell depletion may unmask the correlation between increased

productive CNS HIV infection and clinical neurocognitive disorders (Ellis *et al*., 1997). According to our results, in the final stage of the disease the CNS may be one of the major sites of viral replication, as the $CD4^+$ T-cells have almost completely vanished and the amount of viral antigen in CSF has reached the levels of plasma. Similarly, the viral load in brain tissue of patients with HIVE and of macaques with AIDS was comparable with that in lymphoid organs (Reinhart *et al*, 1997; Wiley *et al*, 1998).

The findings of this study clearly show that not the absolute amount of viral load in the CSF, but the viral antigen contributed by brain cells correlates with the development of neurological disease. This autochthonous virus production must take place for a prolonged period of time to induce overt neurological signs. Further longitudinal human studies and more sensitive methods to quantify the intrathecal viral load are necessary to complete the picture. With the possibility to select for rapid progressing animals (Sauermann *et al*., 1997) and the high neurovirulent potential of SIVmac251MPBMC an animal model is now available to study the pathogenesis of immunodeficiency virus-induced neurological disease in greater detail and to evaluate several therapeutic strategies.

Material and methods

Animals and viruses

Rhesus monkeys (*Maccaca mulatta*) used for this study, were housed at the Deutsche Primatenzentrum, Göttingen, Germany, according to institutional guidelines. They were serologically free of SIV, STLV and SRV. A total of 43 macaques were infected with 100 MID_{50} (50% monkey infective dose) of SIVmac251MPBMC, an SIVmac251-derived virus stock prepared on Rhesus monkey peripheral blood mononuclear cells (MPBMC) (Stahl-Hennig *et al*., 1993), and 35 animals were infected with the viral clone SIVmac239 (n=30) or with closely related pathogenic derivatives of this clone described in other studies (Lang *et al*., 1993, 1997), containing point mutations which did not influence the course of the infection. Animals were monitored clinically, and blood was sampled and CSF collected by suboccipital puncture at regular intervals under anaesthesia. All animals were observed daily by the animal care staff and by a veterinarian on a regular basis. Clinical neurological signs were independently evaluated by two veterinarians. The clinically evident neurological signs observed were ataxia, seizure, apathy not attributable to weakness and opisthotonus. Monkeys were sacrificed when they became moribund or at specified time points according to the experimental schedule. At necropsy, brains from SIV infected rhesus monkeys were thoroughly perfused with 2 l of Hanks buffered salt solution containing 3% foetal calf serum (HBSS). Several slices of brain, 0.5 cm thick, were prepared and replicate slices were processed for either isolation of microglia or *in situ* hybridization. Groups were compared by the Chi square and Fisher's Exact tests of contingency tables.

Preparation of plasma, CSF and microglial cells

Citrated blood samples were subjected to Ficoll-Hypaque (d 1.077) gradient centrifugation (Pharmacia, Freiburg, Germany) in Leucosep-tubes (Greiner, Nürtingen, Germany) and the plasma was stored at -20°C. After removal of CSF-cells by centrifugation at $170 \times g$ for 5 min, the supernatant was collected and refrigerated for further investigations on humoral parameters. In order to minimise the influence of iatrogenic blood contamination during puncture on the parameters determined, CSF samples with more than 5×10^5 erythrocytes/ml (equivalent to a contamination of about 1/10 000) were excluded from further analysis.

Brain haematopoetic cells were isolated from fresh tissue using a Percoll gradient technique (Sedgwick *et al*, 1991) modified for primate brains (Sopper *et al*, 1996). Briefly, after carefully removing the meninges in ice-cold HBSS, pieces of CNS tissue were minced and forced through a 100-mesh metal sieve with the plunger of a 20 ml plastic syringe. The dissociated material was collected by centrifugation at $170 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in DNase/collagenase buffer (41 mM $MgCl_2$, 23 mM $CaCl_2$, 50 mM KCl, 153 mM NaCl) containing 500 U collagenase (Sigma, Deisenhofen, Germany) and 400 U DNase I (Boehringer, Mannheim, Germany) per gram of tissue and digested enzymatically for 60 min at 37°C in a rocking water bath. The suspension was filled up with HBSS to a final volume of 79 ml, mixed with 21 ml isotonic Percoll (pH 7.4, 1.122 g/ml, Pharmacia, Freiburg, Germany) resulting in a density of 1.030 g/ml and transferred to 50 ml centrifuge tubes on top of 5 ml of Percoll at 1.088 g/ml. The tubes were then centrifuged at $1250 \times g$ for 15 min at room temperature. Cells were collected from the 1.088 interface, washed and resuspended in 5 ml HBSS. This cell suspension was layered onto a second Percoll density gradient, composed of five layers with densities of 1.124, 1.088, 1.077, 1.060 and 1.030 g/ml from the bottom of the tube. This gradient was again centrifuged at $1250 \times g$ for 15 min. Cells were collected from the 1.077 interface, washed in HBSS and resuspended in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% FCS (Gibco, Eggenstein, Germany), Napyruvate (1 mM, Biochrom, Berlin, Germany), L-glutamine (2 mM, Biochrom, Berlin, Germany), non essential amino acids (1 : 100, Biochrom, Berlin, Germany) and gentamicine (Boehringer, Mannheim, Germany) (=RPMI+) and counted by trypan blue exclusion using a Neubauer chamber. Immediately thereafter, 1×10^6

isolated cells in 0.5 ml RPMI+ were seeded in 24-cluster plates (Greiner, Nürtingen, Germany). After 20 h of culture, supernatants were collected and stored at −80°C until they were tested for viral antigen by ELISA.

Blood-brain barrier, FACS-analysis
In order to assess the permeability of the blood-brain barrier (BBB), albumin concentrations in blood and CSF were quantified using a standard nephelometric method designed for determination of human albumin (Beckmann, München, Germany) according to the manufacturer's instructions. As a measure for the integrity of the BBB, a quotient of the albumin concentrations in CSF and blood was calculated ($Q_{Alb}=Alb_{CSF}/Alb_{plasma}$). As reported previously, gross or permanent disruptions were not found in any of the animals tested (Sopper *et al*, 1998).

Cells were phenotyped using standard three-colour flow cytometry protocols for Rhesus monkey haematopoetic cells (Sopper *et al*, 1997). Expression of CD3 (FN18, TNO, Rijswijk, The Netherlands) and CD4 (OKT4, Ortho, Neckargemünd, Germany) antigens was used to define $CD4^+$ T-cells, whereas the level of expression of CD11b (44, DPC, Heidelberg, Germany), CD14 (RMO52, Immunotech, Hamburg, Germany) and CD45RA (2H4, Coulter, Krefeld, Germany) was used to differentiate between microglial cells ($CD11b^+$, $CD14^-$, $CD45RA^-$) on the one hand and perivascular or infiltrating macrophages on the other ($CD11b^+$, $CD14^+$, $CD45RA^+$). If the sample permitted, 2×10^4 events were collected for analysis. Otherwise, counting of events was continued until the specimen was exhausted. A minimum of 200 $CD3^+$ T cells in the CSF samples was regarded as necessary for evaluation of the data. As determined by FACS analysis, about 95% of the gradient-purified cells consisted of microglia, the remaining cells being predominately T-cells, some NK-cells and usually less than 3% macrophages. In CSF samples, T-cells represented the majority of infiltrating cells of more than 90%.

Titration of virus, viral antigen and viral RNA
Viral antigen was determined in plasma, cell-free CSF and microglia supernatants using an HIV core antigen capture ELISA (Innogenetics, Zwijndrecht, Belgium) crossreactive with SIV gag. Serial dilutions of supernatant of a persistently SIV-infected C8166 cell culture with known concentrations of viral antigen calibrated with an SIV-specific antigen capture assay (Coulter, Hialeah, USA) were used as standards. Using this standard, a detection limit of 3 pg/ml was determined. All tests were carried out according to the manufacturer's instructions. Infectious virus produced by microglia was titrated on C8166 cells. Briefly, cells were incubated with serial dilutions of culture supernatants in triplicates and were split every third or fourth day. Cultures were monitored for formation of syncytia and cytopathological effects for up to 3 weeks. Measurement of brain viral RNA load was done by quantitative competitive (QC)−RT−PCR. Briefly, 20−30 mg gray matter of snap frozen frontal cortex tissue was disrupted with single use plastic pestle in liquid nitrogen. Tissue lysates were then homogenised using a QIAshredder (Qiagen, Hilden, Germany) spin column. RNA was isolated by RNeasy Mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Samples were frozen at −80°C until QC−RT−PCR was performed as described previously (Haaft *et al*, 1998). Comparisons were performed using Mann Whitney *U*-test. For all statistical analyses, values of samples below the detection limit were expressed as the detection limit.

Histopathology and in situ *hybridization*
Brain sections were immersion-fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with haematoxilin and eosin for histopathology. In addition, SIVE was semi-quantitatively evaluated on H&E sections of complete brains of 16 randomly selected rapid progressors (eight infected with SIVmac239, eight infected with SIVmac251MPBMC). Presence of inflammatory nodules or multinucleated giant cells (MGC) was scored as follows: 1=one or more specific lesions in 1−25% of all brain regions assessed; 2=one or more specific lesions in 26−50% of all brain regions assessed; 3=one or more specific lesions in 51−75% of all brain regions assessed; 4=one or more specific lesions in 76−100% of all brain regions assessed. This resulted in a SIVE score with a range of 0 (neither inflammatory nodules nor MGC throughout the brain) to 8 (both inflammatory nodules as well as MGC in more than 75% of the sections analysed). *In situ* hybridization analyses were performed as described (Czub *et al*, 1996). A transcription vector (pGem-4, Stratagene) containing a gag-specific sequence (527 base pairs) was provided by Dr A Rethwilm (Inst. f. Virologie, Würzburg) and 35S-labelled sense and antisense RNA probes were generated. Brain sections of asymptomatic animals were hybridized with a more sensitive cocktail of riboprobes with additional nef- (800 base pairs) and env-specific (880 base pairs) sequences. Comparison of the sensitivities of the different probes was performed using consecutive sections of several organs. Slides were dipped in 'Ilford K2' photo emulsion and exposed for 8 and 14 days, respectively. Two sections of the temporal brain region encompassing approximately 5 cm^2 were evaluated for viral RNA expressing cells.

Acknowledgements

This work was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und

Technologie, Germany (BMBF 01 KI 9762/5) and in part by Max Plank Forschungspreis (V ter Meulen) and by the Wilhelm Sander Stiftung. We thank Dr ICD Johnston for helpful discussion of the manuscript.

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