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Neuropathology in cats experimentally infected with feline immunodeficiency virus: a morphological, immunocytochemical and morphometric study

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Neuropathological changes have been described associated with feline immunodeficiency virus (FIV) infection. The objective of our study was to characterize the lesions found in the brain and spinal cord of experimentally FIV-infected cats and to quantify, by morphometric analysis, the intensity of gliosis found in these subjects at different time post infection (pi). The brains and spinal cords appeared grossly normal. Gray matter of cortical and subcortical structures showed a moderate to pronounced gliosis particularly in all cerebral cortex and hippocampus. Morphometric analysis demonstrated that GFAP immunoreactivity was markedly higher in infected animals. Gliosis was present 15 days pi and did not appear to progress during the infection, whereas neuronal changes when present were observed only in long-term infected animals (15–23 months pi). In a large proportion of infected cats a diffuse gliosis of white matter and vacuolar myelinopathy was also present. Despite some discrepancies observed between neuropathological changes in FIV-infected animals and HIV-infected individuals, the presence in the cerebral cortex of cats with FIV infection of alterations similar to those observed in AIDS patients demonstrates that FIV is an interesting animal model particularly that may be useful for clarifying the pathogenesis of neuropathological changes associated with HIV infection.

Keywords: feline immunodeficiency virus infection; central nervous system; encephalopathy; immunocytochemistry; morphometry; astrogliosis; vacuolar myelinopathy

Introduction

Dysfunctions of the central nervous system (CNS) characterized by mild cognitive deficits or clinically evident dementing illness have been observed associated with HIV-1 infection (Janssen *et al*, 1989). Even if the exact incidence of these disorders remains uncertain, it has been shown that 30–60% of HIV-infected patients present neurological symptoms (McArtjur, 1987). In other studies the preterminal prevalence of overt AIDS dementia complex was approximately 66% and an additional 25% of preterminal AIDS patients had subclinical form of the disorders (Price and Brew, 1988).

In addition, neuropathological studies have demonstrated that patients with AIDS have a high incidence of a broad spectrum of severe CNS pathological abnormalities including HIV encephalitis or multifocal giant cell encephalitis, HIV leukoencephalopathy, vacuolar encephalopathy and myelinopathy, diffuse poliodistrophy, neuronal loss and cortical dendritic pathology (Budka *et al*, 1987; Gray *et al*, 1991; Masliah *et al*, 1992; Everall *et al*, 1993). These neuropathological changes are thought to arise from both direct infection of CNS tissues by HIV and the host's response to the infection (Atwood *et al*, 1993). The studies to characterize the neuropathogenesis of HIV-1 infection are limited by the difficulty to collect biopsies of brain tissue during the course of AIDS. Present knowledge is therefore predominantly derived from either autopsy or *in vitro* studies. This raises the need for relevant animal

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models that may help to understand the changes occurring during early stage of infection (Vitkovic *et al*, 1995).

Among such models, infection with feline immunodeficiency virus (FIV), a lentivirus of domestic cats belonging to the same subfamily as HIV and SIV (Pedersen, 1993), leads to neurological signs and neuropathological changes similar to those observed in HIV infection (Podell *et al*, 1993). Brain lesions consisting of perivascular mononuclear cell infiltrates, glial nodules and diffuse gliosis, vacuolation and apparent loss of white matter (Dow *et al*, 1990; Hurtrel *et al*, 1992), satellitosis (Podell *et al*, 1993), meningitis and perivascular meningeal calcifications (Hurtrel *et al*, 1992) have been described in FIV-infected cats. Moreover, periaxonal vacuolation of myelin sheaths affecting both dorsal and lateral funiculi and dorsal and ventral nerve roots were also reported (Mitchell *et al*, 1993). These findings were recently supported by previous observations of us demonstrating the presence of diffuse gliosis of gray and white matter in a large proportion of infected animals (Abramo *et al*, 1995). In the present report we further characterized the lesions observed in the brain and spinal cord of cats experimentally infected with two different strains of FIV, using histological, immunocytochemical, ultrastructural, and morphometric techniques at different times post infection (pi).

Results

At postmortem examination no significant gross changes were seen in the brains, spinal cords and meningeal coverings of FIV-infected cats, whereas microscopic brain alterations were commonly present. Neuropathological findings are summarized in Table 1. Cerebral infection by opportunistic agents was never detected. Perivascular sheaths of mononuclear cells were observed only in one subject. The presence of a significant infiltration of lymphocytes involving leptomeninges, myelin pallor and multinucleated giant cells were never detected, while the main feature observed in infected cats was the presence of a moderate to pronounced gliosis involving both gray and white matter (Figure 1a, b). This gliosis was characterized by the presence of swollen GFAP immunoreactive astrocytes.

Table 1 Neuropathological alterations observed in the experimentally infected cats studied

Type of lesion	*Subjects with lesions*
Lymphocytic meningitis	0/15
Perivascular sheaths	1/15
Mild myelin pallor	0/15
Gray matter gliosis	15/15
White matter gliosis	15/15
Vascuolar myelinopathy	5/8

Morphometric analysis confirmed the presence of astrogliosis in the gray matter of cortical structures of experimentally infected animals (Table 2). A marked increase in GFAP reactivity was observed as early as 15 days pi. At this point the increase was higher in the subpial areas than in the deep cortex (Figure 1c, d), and around the blood vessels (Figure 1e, f). In subjects examined between 9 and 23 months pi GFAP reactivity was significantly more severe than in controls. No differences were detected between subjects sacrificed 9 months pi and 23 months pi. Cats infected with Pisa-M2 had higher GFAP reactivity in frontal, parietal and temporal lobes, and a lower GFAP reactivity in the occipital lobe as compared to animals infected with Petaluma but the differences were not statistically significant. Table 3 shows the result of the morphometric studies carried out in the gray matter of subcortical structures. A marked increase of GFAP reactivity was also observed in the caudate nuclei and in hippocampus particularly at 23 months pi. The results of morphometric analysis of GFAP reactivity in the white matter of subcortical structures are presented in Table 4. In the white matter of control cats GFAP reactivity was lower than in the gray matter. Also in the white matter of all lobes the increase of GFAP reactivity was present 15 days pi and was more pronounced at 9 and 23 months pi.

Some subjects sacrificed at 15 months pi and, more frequently, cats examined 23 months pi revealed a diffuse satellitosis in the cerebral cortex, particularly in the temporal and occipital lobes, and the presence of neuronal alterations characterized by vacuolation of the perikarion.

In two of the 14 cats tested for the presence of viral antigen in brain tissues by immunocytochemistry rare cells morphologically consistent with glial cells, were labeled with the anti-p25 monoclonal antibody.

Different degrees of myelin spongy degeneration were observed in both the dorsal and lateral funiculi of infected cats. The results of neuropathological examination of spinal cords are reported in Table 5. No spinal cords changes were observed in the control cats except for occassional multifocal splitting of myelin lamellae. Moderate to diffuse splitting, characterized by lamellar separation of the myelin sheath, and the presence of intramyelin and periaxonal vacuoles, accompanied by whorl-shape myelin debries among the nerve fibres, were the main features in cats with FIV infection (Figure 2a, b). Splitting of the myelin sheaths was observed in all infected animals. The dorsal funiculus appeared more involved. Intramyelin vacuoles were present in five out of the eight experimentally infected animals examined. These subjects frequently showed partially demyelinated axons, named naked

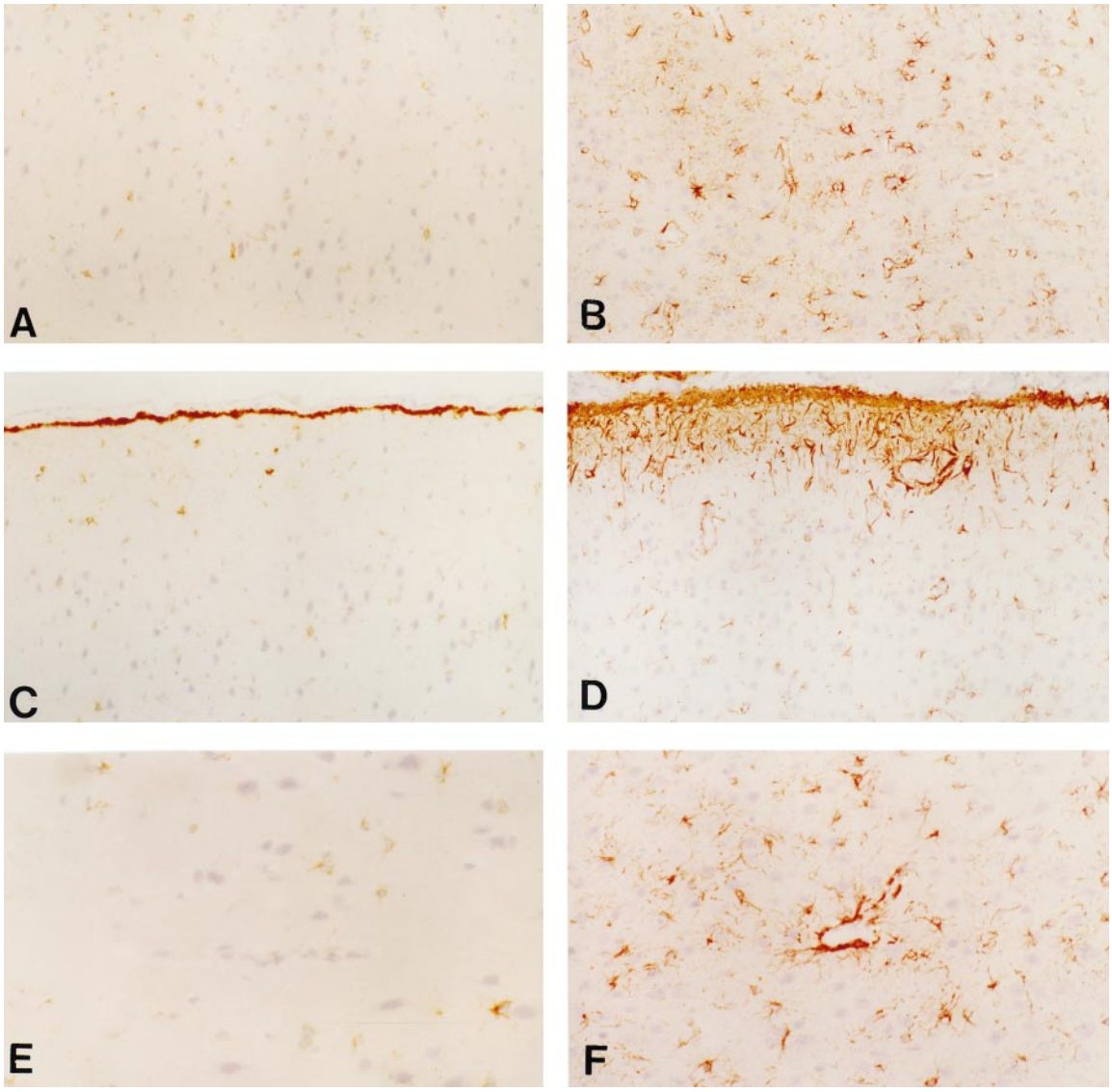

Figure 1 GFAP immunoreactivity in the gray matter of cortical structures of SPF control (**A**, **C** and **E**) and FIV-infected cats (**B**, **D** and **F**). (**A**) Only rare astrocytes are present in cortical areas of a control cat (×120); (**B**) Cat infected with FIV Pisa-M2 sacrificed 15 months post-infection. Presence of severe gliosis characterized by the presence of numerous hypertrophic GFAP-reactive astrocytes (×120). (**C**) Subpial astrocytes in a control subject (×120); (**D**) Cat infected with FIV Pisa-M2 sacrificed 15 days post-infection. The increase in GFAP-reactivity was evident in subpial areas (×120). (**E**) Absence of GFAP reactivity around a blood vessel in a control cat (×180). (**F**) Cat infected with FIV Pisa-M2 sacrificed 15 days post-infection. GFAP-reactive astrocytes around a blood vessel (×180).

axons (Figure 2c). They were characterized by focal myelin breakdown, small vacuoles at the point of myelin sheath loss and unscathed axoplasm. The only axonal involvement was a mild axonal distortion due to myelin spongy degeneration and was observed only occasionally. Macrophages were present in association with naked axons or closely applied to the vacuoles. Lipid-laden macrophages were occasionally seen (Figure 2d).

Discussion

The lesions observed in experimentally FIV-infected cats included the presence of perivascular sheath, vascular myelopathy, and diffuse gliosis of the gray and white matter of telencephalic structures. The neuropathological changes observed in the present study were similar to those described in previous studies (Dow *et al*, 1990; Hurterl *et al*, 1992; Podell *et al*, 1993; Abramo *et al*, 1995).

Even though comparison of neuropathological changes between natural and experimental infection evidenced some differences, a part at least of these alterations were observed in both groups. The severity of diffuse gliosis of the gray and white matter and vacuolar myelinopathy varied but did not differ in cats with natural or experimental infection, whereas meningeal involvement and perivascular sheaths and myelin pallor were mainly

Table 2 Morphometric analysis of GFAP-reactivity in the gray matter of cortical structures of experimentally infected and control cats[a]

Cats (number examined)	*Cerebral lobes*						
	Frontal			*Temporal*		*Occipital*	
	Subpial	*Deep*	*Parietal*	*Subpial*	*Deep*	*Subpial*	*Deep*
SPF control cats (*n*=5)	241±320	153±224	528±454	345±360	308±207	631±365	445±303
15 days pi (*n*=2)	2110±942	1068±1038	1494±891	1360±391	1756±398	5094±2075	2217±1478
9 months pi (*n*=5)	511±331***	746±604***	1778±877***	1165±512***	1238±507***	1920±747***	2265±662***
23 months pi							
FIV Petaluma (*n*=5)	591±575***	1035±770***	1351±666***	1092±906***	1192±879***	3399±1536***	3242±2146***
FIV Pisa M2 (*n*=3)	1520±601***	1998±768***	2308±877***	2282±712***	1848±825***	2543±1127***	2195±638***

[a] GFAP reactivity is expressed in μm^2; pi=post-infection. ***P<0.001

Table 3 Morphometric analysis of the GFAP-reactivity in the gray matter of caudate nuclei and hippocampus of experimentally infected and control cats.[a]

Cats (number examined)	*Caudate nuclei*	*Hippocampus*
SPF control cats (*n*=5)	349±291	565±398
15 days pi (*n*=2)	702±726	1888±785
9 months pi (*n*=5)	2034±1286***	1622±622***
23 months pi		
FIV Petaluma (*n*=5)	1199±1390***	2485±891***
FIV Pisa M2 (*n*=3)	1396±844***	2451±882***

[a] GFAP reactivity is expressed in μm^2; pi=post-infection. ***P<0.001

observed in naturally infected subjects (Abramo *et al*, 1995). These findings confirm the presence in the subjects with natural infection of more severe and widespread brain changes than in experimentally infected subjects (Hurtrel *et al*, 1992; Podell *et al*, 1993). Possible explanations may be the fact that naturally infected animals had a longer lasting of the infection or had reached a more advanced stage of illness.

In spite of these differences previous studies of us and other groups demonstrate that at least some neuropathological alterations, such as the presence of perivascular infiltrates, diffuse gliosis and vacuolar myelinopathy, occurr in experimentally infected SPF cats negative for opportunistic infections and in the absence of therapeutic treatments. On the basis of these findings, these neuropathological changes may be considered morphological correlates of FIV infection of the CNS. To corroborate this hypothesis we revealed the presence of viral core antigen within the brain tissue of infected animals, even though the viral load appeared to be low at least on the basis of immunocytochemistry.

Among the lesions described associated with FIV infection, diffuse gray and white matter gliosis and vacuolar myelinopathy showed a higher prevalence. In our study the histological diagnosis of gliosis was further confirmed by morphometric analysis of GFAP immunostained brain sections that revealed a marked increase in the size of astrocytes, a change commonly believed to reflect gliosis (Cunha *et al*, 1993). Preliminary results suggest that the increase of GFAP immunoreactivity is due to astrocytic hypertrophy more than to astrocytic proliferation.

Table 4 Morphometric analysis of the GFAP-reactivity in the white matter of cortical structures of experimentally infected and control cats.[a]

Cats (number examined)	*Cerebral lobes*			
	Frontal	*Parietal*	*Temporal*	*Occipital*
SPF control cats (*n*=5)	112±111	187±107	263±197	161±77
15 days pi (*n*=2)	1905±691	3877±1318	2137±538	1444±682
9 months pi (*n*=5)	926±589***	1904±1076***	1510±1016***	3045±755***
23 months pi				
FIV Petaluma (*n*=5)	224±187**	1644±671***	1674±876***	1210±662***
FIV Pisa M2 (*n*=3)	835±362***	3473±910***	1763±1003***	2845±617***

[a] GFAP reactivity is expressed in μm^2; pi=post-infection. **P<0.01; ***P<0.001

Table 5 Neuropathological spinal cord changes in experimentally infected and control cats[a]

	Control cats				*15 days pi*		*9 months pi*			*23 months pi*		
Cats number	*159*	*160*	*161*	*165*	*152*	*151*	*172*	*117*	*118*	*119*	*120*	*121*
Dorsal funiculus												
Splitting	+	+	0	0	0	++	++	+	+	+++	+	+
Vacuoles	0	0	0	0	0	++	++	+	0	++	0	0
Debries	0	0	0	0	+	+	+++	+	+	+	+	+
Naked axons	0	0	0	0	+	+	++	0	0	++	0	+
Lateral funiculus												
Splitting	+	0	+	0	+++	++	+++	0	0	+++	0	+
Vacuoles	0	0	0	0	++	++	++	0	0	++	0	0
Debries	0	0	0	0	++	0	++	+	0	+	0	+
Naked axons	0	0	0	0	0	++	0	+	0	+	0	0

[a] Severity of lesions: 0 no lesion, + rare, ++ scattered, +++ numerous

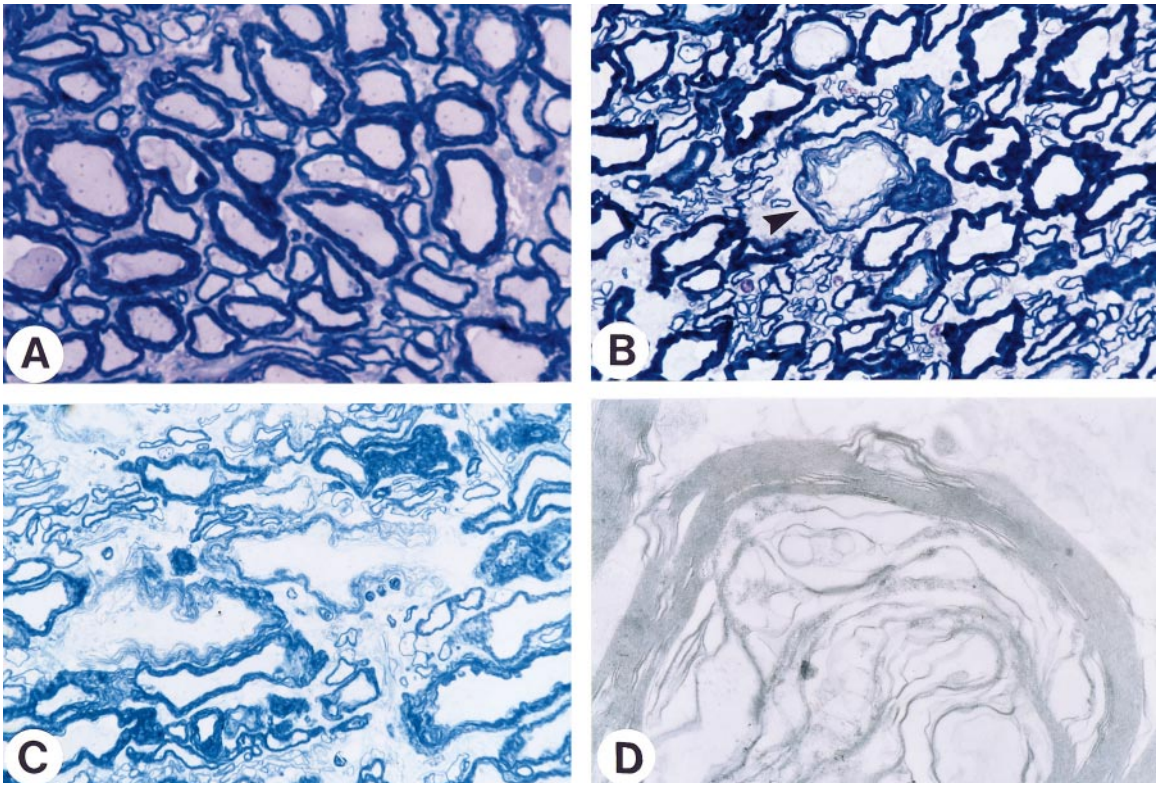

Figure 2 **(A)** Spinal cord white matter (transverse section) of a SPF uninfected cat. Myelin sheaths and axons are well preserved (0.5 μm-thick section stained with methylene blue-azur II, $\times$100). **(D)** Spinal cord white matter (transverse section) of a FIV-infected cat sacrificed 15 months post-infection. A few myelin sheaths show a poor compactness of the lamellae (arrow head), in one fiber small intramyelin vacuoles are present (arrow) (0.5 μm-thick section stained with methylene blue-azur II, $\times$100). **(E)** Spinal cord white matter (longitudinal section) of a FIV-infected cat sacrificed 24 months post-infection. Marked myelin sheath abnormalities are present, characterized by separation of myelin lamellae and focal myelin breakdown. Partially naked axons are also evident (0.5 μm-thick section stained with methylene blue-azur II, $\times$100). **(F)** Electron micrograph from the same cat in **(E)**. Intramyelin periaxonal swelling and vacuoles are present. The outer border of the myelin sheath still shows normal intraperiod lines ($\times$4500)

Diffuse gliosis of gray matter in cortical and subcortical structures found in FIV-infected cats, has been also frequently detected in AIDS patients, described as a diffuse pathology of the gray matter in cerebral cortex, basal ganglia, and brain stem nuclei and referred to as diffuse poliodystrophy (Budka *et al*, 1987). In FIV infected animals we also observed the presence of diffuse white matter gliosis sometimes associated with mild damage of the white matter due to myelin loss in the absence of inflammatory infiltrates, a lesion similar to the leukoencephalopathy frequently described in AIDS patients (DeGirolami *et al*, 1990; Budka, 1991). In FIV-infected cats these changes were not accom-

panied by the multinucleated giant cells that are instead observed in HIV-infected humans (Hurtel *et al*, 1992). In experimentally FIV-infected animals gliosis appeared early (15 days pi) and did not change markedly in later stages of infection, confirming previous observations (Hurtel *et al*, 1992). Thus, on the basis of our findings gliosis seems to occur very early in the course of the illness. Neuronal changes, characterized by vacuolation of perikarion, developed later and were detected only in animals sacrificed 15 months pi or later.

The lesions observed in cats infected with two different viral strans did not differ quantitatively, but cats infected with Pisa-M2, a low passage isolate, showed a more marked gliosis than those infected with the Petaluma isolate grown in persistently infected lymphoid cells (Bendinelli *et al*, 1995) and known to possess a reduced pathogenicity.

Our studies indicate the occurrence of a spinal cord involvement in naturally and experimentally FIV-infected cats. The spinal cord changes observed in FIV infection are similar, although with some differences, to those described in HIV-infected patients. In HIV infection the main feature is vacuolar myelin swelling (Tan *et al*, 1995). In our study the most prominent histopathological features were the presence of different degrees of myelin sheath splitting and intramyelin vacuoles. Almost all the cats showed myelin sheath splitting and in 5 of these animals intramyelin vacuoles were observed. Therefore a possible progressive correlation between *splitting* and vacuolar lesions is suggested. This data might indicate that the pathological changes of the spinal cord start with separation of myelin lamellae and is then followed by vacuolation as the lesion advances. Occasional multifocal myelin splitting was also observed in SPF control cats, but the lesion was always mild.

The presence of naked axons has never been described in HIV infection while in our study was sometimes observed in the dorsal funiculus of infected cats. In AIDS-associated vacuolar myelopathy the prominence of macrophages in mild and moderate vacuolar myelopathy suggests an early involvement of macrophagic cells in the pathogenesis of myelin damage (Tan *et al*, 1995). Unfortunately the limited number of cases in our experiment groups does not allow to validate the hypothesis proposed for HIV infection, even though the occasional observation of macrophages around myelin sheath suggests that these cells are in some way involved also in FIV neuropathology. The absence of axonal degeneration, found only in some severely HIV infected patients (Petito *et al*, 1985, 1994), confirms that white matter changes are not secondary to an axonopathy and likely indicates that the cats examined in our study had not reached an advanced stage of illness. This fact might explain the absence of clinical neurologic abnormalities. Though failure to detect clinical signs of CNS dysfunction probably also depends on an inability to identify subtle neurologic manifestations (e.g. behavioral changes) in cats. Moreover, neurological abnormalities may occur secondarily to opportunistic CNS infections that however were never detected in this and previous studies (Dow *et al*, 1990).

In conclusion, our results confirm the presence of a pattern and neuropathological changes associated with FIV infection. These alterations are well characterized and are in part similar to those associated with HIV-1 infection of human. Interestingly, the lesions observed in FIV-infected cats resembled those observed in hemophiliacs with HIV-1 infection. These patients often die earlier than HIV-1-infected non-hemophiliacs and have fewer CNS opportunistic infections and a lower prevalence of HIV encephalopathy. In contrast, they show a higher prevalence of nonspecific features, such as perivascular lymphocyte cuffing, scattered microglial nodules, mild diffuse myelin pallor with mild astrocytosis, and subpial astrocytosis in the cerebral cortex (Esiri *et al*, 1989; Esiri, 1993). Similar alterations were also observed in asymptomatic subjects (Gray *et al*, 1996). In both groups HIV proteins are seldom detected in the brain by immunocytochemistry (Achim *et al*, 1991), while in AIDS patients with HIV encephalopathy high loads of virus are constantly found (Sinclair *et al*, 1994). Also in FIV infection a mild CNS involvement seems to be associated with a low viral load. Nevertheless, this finding should be confirmed by further immunocytochemical or *in situ* hybridization studies.

Materials and methods

Animals

The 20 specific pathogen free (SPF) cats used were females aged between 1–4 years. Fifteen subjects were infected with the Pisa-M2 (seven animals) or the Petaluma (eight animals) strains of FIV. The subjects infected with the Pisa-M2 isolate were inoculated intravenously with 2 ml of freshly collected blood containing the virus propagated *in vivo* by monthly passages in SPF cats (Matteucci *et al*, 1993), while the cats infected with Petaluma isolate were inoculated with culture supernatant of persistently infected FL4 cells (Yamamoto *et al*, 1991) containing approximately 20 DIC_{50}. Five FIV-seronegative SPF cats were used as controls. Throughout the observation period, infected and control cats were housed in isolation units (Techniplasts, Gazzada, Va, Italy) in the Retrovirus Center of the University of Pisa and were observed daily. Physical examination including measurements of body weight and clinical neurological investigations, were performed weekly for the first 2 months pi and then monthly. The latter consisted of an

overall assessment of their mental state, behavior, gait, posture and movement. All infected cats seroconverted in 4 to 6 weeks: the presence of specific anti-FIV antibodies was determined using an in-house enzyme linked immunosorbent assay (Lombardi *et al*, 1994) and was confirmed by Western blot analysis on electrophoresed FIV proteins from gradient-purified tissue culture-growth virus. These animals presented a reduction in the number of circulating $CD4^+$ lymphocytes that in Pisa-M2 infected cats was by almost two-thirds in 1 year. At 0.5, 9, 15 and 23 months pi, groups of animals were induced to a deep anesthesia and necropsied. At the time of sacrifice all the experimentally infected animals showed no obvious neurological signs.

Tissue processing
Brains and spinal cords were obtained immediately after necroscopy and fixed in 10% buffered formalin (pH 7.4), embedded in paraffin, and stained by routine neuropathological stains. Large size uniemisheric blocks and other small blocks from representative parts of the brain and spinal cord were examined. Studied areas included: frontal, parietal, temporal, and occipital lobes, basal nuclei, anterior and posterior thalamus, hippocampus, genicolate nuclei, mesencephalon, pons, cerebellum and caudal medulla. To exclude opportunistic infections selected sections from the CNS were also stained with Gram, Ziehl-Nielsen, periodic acid-Schiff, alcian blue and Grocot stains. For demonstration of astrocytes paraffin sections of selected areas were stained with polyclonal antiserum directed to the GFAP (Biogenex Lab, San Ramon, CA, USA) in a Shandon Sequenza immunostaining method as previously described (Abramo *et al*, 1995). Paraffin embedded tissues were also examined for the presence of FIV p25 viral core antigen using a streptavidin-biotin peroxidase labeled complex technique and monoclonal antibodies against FIV p25 antigen (Lombardi *et al*, 1994). Formalin-fixed, paraffin embedded FIV-infected FL4 cells and tissues from FIV-negative SPF cats served as positive and negative controls, respectively. A non-related monoclonal antibody was included in each assay as further negative control.

Morphometry
GFAP immunostained sections containing gray and white matter from frontal, parietal, temporal, and occipital lobes were examined. Caudate nuclei were considered as representative of basal nuclei. Anterior and posterior thalamus and hippocampus were also used for morphometrical analysis. Ten microscopic fields ($\times 25$ objective) were captured by a television camera and projected to a monitor screen of an automated image analysis system (Quantimet 500 MC, Leica, Germany). GFAP-reactive astrocytes were counted and data were given in square microns (μm^2).

Statistics
Normality of distribution was tested with the Kolmogorov-Smirnov's test. Group means $\pm$ standard deviations were calculated. Analysis of variance was carried out and significance of differences between groups was evaluated with the Bonferroni's test.

Ultrastructural study of spinal cord
The spinal cords of eight experimentally and three control cats were examined. The experimentally infected animals were inoculated with the Petaluma strain and sacrificed at 15 days (two cases), 9 months (one case) and 23 months pi (five cases). The thoracic spinal cord was removed and samples of funiculi and lateral columns were dissected and fixed by immersion in 10% buffered formalin solution. Transversally and longitudinally oriented blocks were embedded in paraffin to better evaluate myelin and axonal morphology and examined using routine neuropathological stains.

For electron microscopy fresh samples of funiculi and lateral columns of thoracic spinal cord were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C. Samples of gray and white matter were post-fixed in 1% buffered osmium tetroxide and embedded in Epon. Transverse and longitudinal 1 μm sections were stained with methylene blue-azur II solution. Ultrathin sections were double stained with 2% uranyl acetate and lead citrate and observed with a Philips CM-10 transmission electron microscope.

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