Virus load and neuropathology in the FIV model

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The FIV (feline immunodeficiency virus) induces in cats brain changes presenting similarities with those observed in human immunodeficiency virus infection. This FIV model was used to study the relationship between viral load in brain, in lymphoid organs and central nervous system (CNS) changes during the early and late stages of infection. Early brain changes were analyzed in animals experimentally infected with two different FIV isolates and sacrificed at 7 and 15 days, 1, 2, 6, and 12 months post inoculation (p.i.). Late CNS abnormalities were analyzed in naturally FIV-infected cats referred to the Veterinary School of Nantes. For each animal, one cerebral hemisphere was fixed and examined using routine techniques. The characterization of FIV replicating cells by in situ hybridization was performed on the other half frozen hemisphere on sections performed in the anterior and the median regions of the brain. During the early stages of infection, moderate gliosis with glial nodules and sometimes white matter pallor and meningitis were associated with few infected cells scattered in the brain. Infection was an early event as infected cells could be detected in brain at 7 p.i. For each cat, these findings were found identical in the two analyzed areas. During the late stages, brain lesions and the number of virus replicating cells increased especially in animals with perivascular infiltrates. The multinucleated giant cells encephalitis was never observed and the number of FIV replicating cells scattered in the whole brain was always low. This discrepancy between the number of replicating cells and the brain lesions, corroborates the hypotheses suggesting that brain injuries may be mediated via diffusive factors and amplification processes through cytokine cascades and cell activations.

Keywords: FIV; neuropathology; viral load

Introduction

Neurological impairments related to HIV (Human Immunodeficiency Virus) infection are the most severe complication of AIDS. One third of adults and half of children with AIDS will develop neurological symptoms directly attributable to HIV, and at autopsy 80% of AIDS patients present central nervous system (CNS) abnormalities (Henin et al, 1987; Elder and Sever, 1988; Michaels et al, 1988; Price et al, 1988; Budka et al, 1991; Gray et al, 1988, 1991; Janssen et al, 1991; Gendelman et al, 1994). The etiopathogenesis of HIV dementia occurring in late stages is unknown, and could be due to a primary effect of the virus itself or be induced by a host's response to infection (Dubois-

Dalcq et al, 1990; Sharer, 1992; Spencer and Price, 1992; Dickson et al, 1994; Nottet et al, 1995). To date, it has been clearly shown that HIV-1 elicits brain dysfunction in the absence of conclusive productive infection of neurons, astrocytes and oligodendroglia (Koenig et al, 1986; Vazeux et al, 1987; Chakrabarti et al, 1991) and the central question is to know whether a relatively small number of infected cells can cause this severe neurologic dysfunction. Neurobehavioral and pathological data (Carne et al, 1985; Ho et al, 1985; Resnick et al, 1985, 1988; Goudsmit et al, 1986; Hollander et al, 1987a, b; Elder and Sever, 1988; Budka et al, 1991; Davis et al, 1992) suggest that CNS becomes infected early during the primary infection. However, neuropathogenesis of HIV infection is difficult to investigate because the brain parenchyma is not accessible to sampling, therefore the major knowledge of the neuropathogenesis of HIV 1 infection originates mainly from either

postmortem or *in vitro* studies. Tissue culture systems have significant limitations and therefore could hardly reflect the complexities of cell to cell brain interactions *in vivo*. The analysis of the early stages of the CNS infection and its progression to the terminal phase in brain only infected with HIV is extremely difficult in man but fundamental in understanding the HIV CNS pathophysiology. Moreover the delay between death and the harvest of brain parenchyma in man is often long making the possibility of detection of viral or cellular RNA difficult.

This raises the need for animal models of HIV infection for the understanding of neuropathogenesis of the HIV infection (Persidsky *et al*, 1995). Cats infected by feline immunodeficiency virus (FIV), a lentivirus discovered in 1986 by Pedersen (Pedersen *et al*, 1987), could represent a useful animal model because of the animal size and the non pathogenicity of FIV for man. FIV is known to cause in cats pathogenesis similar to those observed during HIV infection (Pedersen *et al*, 1987, 1989; Yamamoto *et al*, 1988; Ackley *et al*, 1990; Novotney *et al*, 1990; Barlough *et al*, 1991; Callanan *et al*, 1992; Beebe *et al*, 1994). Neurological involvement is also noted with FIV infection such as central sensory electrophysiological changes and sleep disturbance (Pedersen *et al*, 1987; Dow *et al*, 1990, 1992; Hurtrel *et al*, 1992; Podell *et al*, 1993; Phillips *et al*, 1994; Prospero-Garcia *et al*, 1994; Abramo *et al*, 1995; Darko *et al*, 1995). FIV was recovered from cerebrospinal fluid (CSF) and brain tissue of naturally and experimentally infected cats (Yamamoto *et al*, 1988; Dow *et al*, 1990). Seroepidemiological surveys have shown that some FIV-infected cats develop behavioral abnormalities and encephalopathy that resemble HIV dementia (Podell *et al*, 1993). CNS entry for FIV, like HIV, seems to be an early event as the virus could be isolated from primary culture from brain and CSF (Dow *et al*, 1990) and CNS lesions has been described as early as 1 month following FIV intravenous (IV) inoculation (Hurtrel *et al*, 1992). This model was used to study the relationship between virus load in brain, in lymphoid organs and brain changes during the early and late stages of infection.

Results

Early stages

Two groups of animals were infected with the Petaluma or the Envnip isolate inoculated by IV route and sacrificed at 7 and 15 days, 1, 2, 6 and 12 months p.i. For each animal, complete necropsy and histopathological examinations were performed, and a systematic study was carried out on the brain. One cerebral hemisphere was fixed in formalin and examined after usual staining and the other half was used for *in situ* hybridization on sections performed in the anterior and the median

areas of the brain. The detection of the FIV, with the antisense *Pol* gene probe, showed the classic labeling in spots of greater or lesser intensity (Figure 2). This corresponded to the level of viral replication in cells and were identical to the labeling of the isolated lymph node (LN) cells infected *in vitro*, which were used in all the experiments as positive control for verification of the sensitivity of the technique and which remained identical throughout the experiments. Isolated FIV infected cells were found scattered in all the brain, generally in the white matter but few infected cells could be detected in the grey matter. The infected cells producing the virus were counted by 50 mm^2 of surface area in brain and a minimum of ten sections were analyzed and the mean calculated. In each animal, we did not see any significant difference between the anterior and the median sections and therefore the results were grouped together and presented in Figure 1. The two viruses tested gave
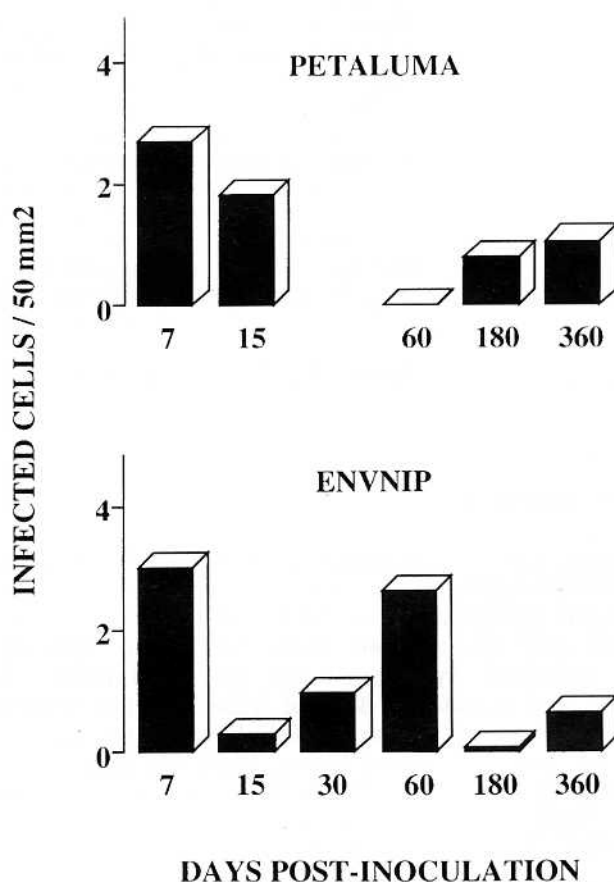
Figure 1 Quantification of FIV infected cells by *in situ* hybridization, in brains of eleven cats experimentally inoculated IV with the Envnip or the Petaluma strain. One animal was sacrificed at 7, 15, 60, 180 and 360 days p.i. in the Envnip infected group, in the Petaluma group one animal sacrificed at 30 days was added. FIV infected cells were counted on a minimum of ten complete sections performed in the anterior and the median regions of the brain and results expressed as the mean by 50 mm^2.

the same low viral load during the early stages of infection with a relative peak at 7 days p.i. These results corroborate the early passage of virus at 7 and 15 days p.i., as described in SIV model. During this primary infection, *in situ* hybridization features suggest that the virus enters the brain via infected
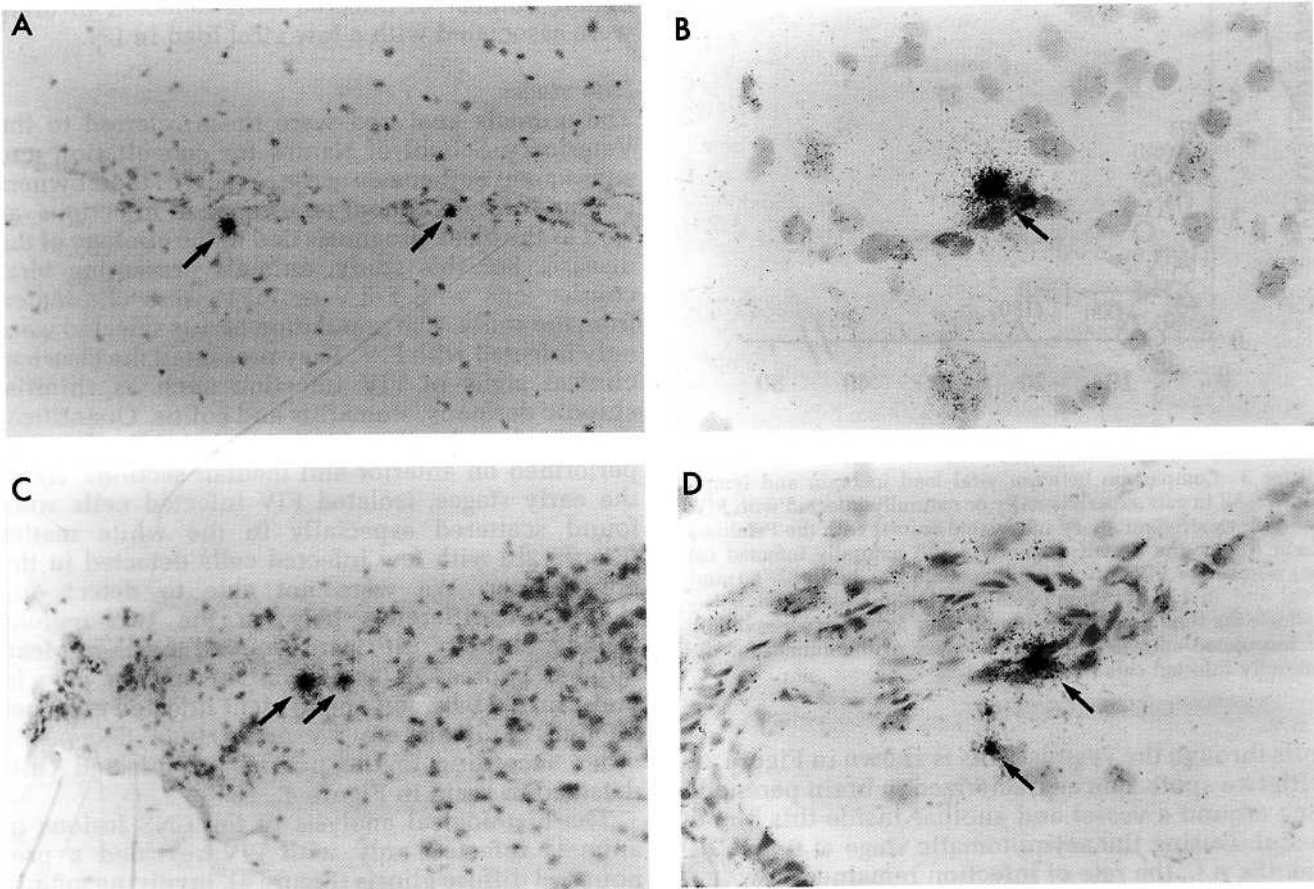

Figure 2 Infected cells characterized by *in situ* hybridization, in brains of cats experimentally or naturally infected with FIV. (A) Perivascular and intravascular infected cells detected in white matter of a cat infected IV with the Petaluma strain at 7 days p.i. (× 108). (B) Infected cells associated with diffuse gliosis in white matter of a cat naturally FIV infected (animal 431) (× 324). (C) Infected cells in a glial nodule in white matter of a cat naturally FIV infected (animal 654) (× 108). (D) Perivascular and intravascular infected cells detected in white matter of a cat naturally FIV infected (animal 431) (× 324).

Table 1 Neuropathological analysis of experimentally FIV infected animals

Days post-inoculation	Envnip						Petaluma				
	7	15	30	60	180	360	7	15	60	180	360
Neuropathol. findings											
gliosis	+	++	++	+++	++	++	++	++	++	++	++
glial nodules		++	+	+	+	+	++	++	++	+	+
white matter pallor	+	+			+	+	++	+			
meningitis	+					+					
Pathological findings											
lymphadenopathy	+	++	++	++	+++	++	++	+++	+	+	++

For each animal, the findings are summarized in one column. Two groups of animals were inoculated by IV route with the Envnip strain (left) or the Petaluma strain (right) and sacrificed at 7, 15 days, 1, 2, 6, or 12 months p.i. Frequency and severity of lesions: (gliosis, glial nodules, white matter pallor) (+) rare and moderate, (++) frequent and moderate, (+++) frequent and marked, (meningitis) (+) moderate. Two lymph nodes (LN) (medial retropharyngeal and superficial cervical LN) were analyzed and compared to three non infected controls. The LN presented a marked follicular reaction: follicular areas larger than control, germinal centers small and always present (+), marked development of follicular areas and germinal centers (++), significant development of follicular areas and germinal centers (+++)
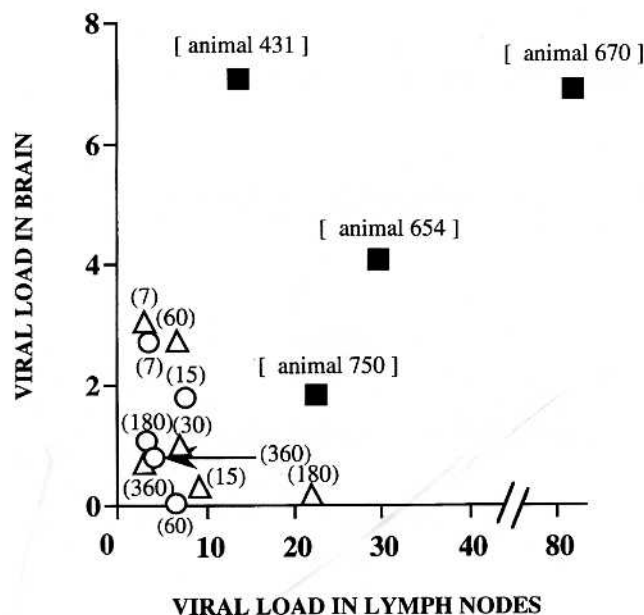
Figure 3 Comparison between viral load in brain and lymph nodes (LN) in cats experimentally or naturally infected with FIV. For each experimentally IV inoculated animal with the Petaluma strain (○) or the Envnip strain (△) and naturally infected cat (■) number of FIV infected cells counted in brain (in 50 mm² area) and in LN (in 5 mm² area) is plotted together. Brackets indicate the time post inoculation in days for the experimentally IV inoculated animals ( ) or the identity of the animal for the naturally infected cats [ ].

cells through the vessels. This is shown in Figure 2a with two spots, one characterized in brain parenchyma around a vessel and another inside this blood vessel. During the asymptomatic stage at 6 and 12 months p.i., the rate of infection remained low.

The results of the histopathological analysis performed on the other formalin fixed hemisphere are summarized in Table 1. As for the viral load, we did not detect any difference between the two FIV-strains tested. The animals showed moderate gliosis, involving both the white and the grey matter generally associated with microglial nodules. Focal white matter pallor (WMP) was appreciated on myelin stain and could be detected during the early stages at 7 and 15 days p.i. and later at 180 and 360 days p.i. with the Envnip strain. These lesions were present in the whole brain. Two animals showed discrete meningitis characterized by infiltration with mononuclear cells.

For each animal two LN, the medial retropharyngeal and the superficial cervical LN, were analyzed and compared to those in three non infected controls. All animals presented follicular hyperplasia (Table 1) and the number of infected cells characterized by *in situ* hybridization was quantified. For each cat, the mean number of infected cells counted in LN and in brain were plotted on the same graph (Figure 3). Some correlation appeared in animals infected with the

Envnip isolate at 30 and 360 days p.i. and with the Petaluma isolate at 180 and 360 days p.i. Nevertheless one animal with numerous infected cells in LN presented a very low viral load in brain at 180 days p.i. and during the early stages at 7 days p.i. a higher number of infected cells detected in brain were associated with a low viral load in LN.
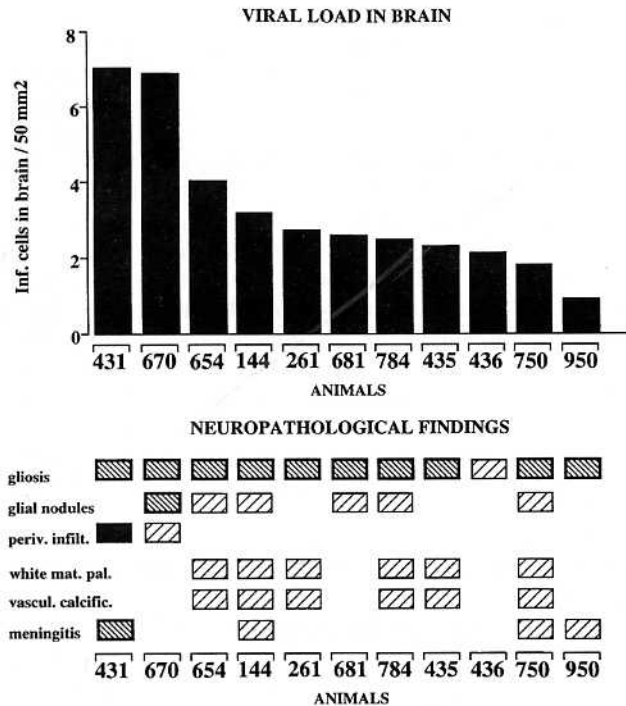
Late stages
The animals analyzed were those referred to the Veterinary School of Nantes for consultation and subsequent euthanasia at the request of their owners because of the frequent recurrence of infections, as well as the bleak prognosis tied to the etiology of the disease. For this study, animals presenting viral coinfections with FeLV or FIPV were eliminated from the study. The population of cats selected were only infected with FIV. They presented the classical clinical signs of FIV infection such as rhinitis, chronic dermatis, stomatitis and colitis. Quantification of infected cells using *in situ* hybridization was performed on anterior and median sections. As in the early stages, isolated FIV infected cells were found scattered especially in the white matter (Figure 2b) with few infected cells detected in the grey matter. We were not able to detect any significant difference between the two regions tested and the results were grouped together. Mean number expressed by 50 mm² of surface area in brain varied between one to eight infected cells per 50 mm². Animals are presented in a decreasing order according to the number of infected cells detected in brain in Figure 4.

The histological analysis of the CNS lesions in animals infected only with FIV revealed a pronounced diffuse gliosis (Figure 4), involving mainly the white matter. Six animals had glial nodules located in the cortical or periventricular region often associated with infected cells (Figure 2c). Perivascular infiltrates were relatively uncommon (animals 431 and 670). They were associated in one cat with meningitis and infected cells (Figure 2d). Small vascular calcification were found in the leptomeninges associated with focal WMP. Meningitis was relatively frequent, including congestion, slight oedema and moderate infiltration of mononuclear cells.

As the owners generally did not allow to extend clinical exploration before euthanasia, complete necropsy and histopathological examinations were performed to characterize disease evolution on each animal. The major pathological findings including chronic renal insufficiency, stomatitis, colitis, rhinitis, dermatitis were associated with thinness and sometimes cachexia (Table 2). The immunodeficiency status of each animal was estimated by LN analysis, severe immunodeficiency was associated with hypoplasia and preAIDS syndrome with marked hyperplasia. The results are summarized in Table 2. The two higher numbers of infected cells

counted in the brain were associated with perivascular infiltrates. Moreover cats with higher viral load in brain presented numerous associated pathological findings including lymphadenopathy

characterized by a severe hypoplasia or hyperplasia. Quantification of infected cells in LN was performed in four cats presenting a high (431, 654, 670) or a low number (750) of infected cells in brain. Mean numbers were plotted on the same graph (Figure 3). Results show that there is some correlation between viral load in LN and brain for three cats (654, 670, 750) but not for the animal presenting a drastic LN hypoplasia (431).

Figure 4 Viral load in brain and neuropathological findings in the naturally FIV infected cats. Animals are presented in the same order than in Table 2 and findings concerning each animal are summarized in one column. Frequency and severity of lesions: gliosis: (▨) frequent and moderate, (▧) frequent and marked; glial nodules: (▨) rare and marked, (▧) frequent and marked; perivascular infiltrates: (▨), frequent and moderate (■) frequent and severe; white matter pallor (▨) rare and moderate; vascular calcification: (▨) rare and moderate; meningitis: (▨) moderate, (▧) marked.

Discussion

FIV induces in cat brain changes presenting similarities with those observed in HIV infected individuals (Pedersen et al, 1987; Dow et al, 1990, 1992; Hurtrel et al, 1992; Podell et al, 1993; Prospero-Garcia et al, 1994; Darko et al, 1995) at comparable stages of retroviral infection and therefore it was used to study the relationship between viral load in brain, in lymphoid organs and brain changes in a longitudinal analysis.

During the early stages of infection encephalopathy, lesions and viral load in brain were identical in the two viruses tested. As described in SIV model (Chakrabarti et al, 1991; Hurtrel et al, 1993), brain infection was found to be an early event as infected cells could be detected at 7 days p.i. This early infection corroborates the studies suggesting that FIV might enter the CNS shortly after infection (Pedersen et al, 1987, 1989; Yamamoto et al, 1988; Ackley et al, 1990; Novotney et al, 1990; Barlough et al, 1991; Callanan et al, 1992; Beebe et al, 1994). These results support our previous study (Hurtrel et al, 1992) which showed that the same lesions were obtained during initial stages, after inoculation of FIV administered either by intracerebral or IV route. It also corroborates studies in which the virus was recovered by primary culture from cerebral cortex, caudate nucleus, midbrain, cerebellum, caudal

Table 2 Lymphadenopathy and pathological findings of the naturally FIV infected cats studied

	Animals		Lymphadenopathy		
	Sex	Age	Hyperplasia	Hypoplasia	Pathological findings
431	M	10		+++	cachexia, chronic renal insufficiency, chronic dermatitis
670	M	7	+++		rhinitis, stomatitis, chronic dermatitis
654	M	10		++	cachexia, chronic renal insufficiency, stomatitis
144	M	8	+++		colitis, thinness
261	M	12	++		stomatitis, thinness
681	M	2		+	chronic renal insufficiency, chronic dermatitis
784	M	6		++	cachexia, chronic renal insufficiency, rhinitis
435	M	10	++		thinness
436	M	9		+	rhinitis
750	M	5	++		rhinitis
950	M	2,5	++		colitis, rhinitis

LN were analyzed as described in Table 1. LN presenting follicular hyperplasia: (++) marked development of follicular areas and germinal centers, (+++) significant development of follicular areas and germinal centers; LN presenting follicular hypoplasia: (+) moderate atrophy of follicular areas, (++) marked atrophy of follicular areas, (+++) severe atrophy of follicular areas

brainstem and cerebrospinal fluid, early after IV inoculation (Yamamoto et al, 1988; Dow et al, 1990). Both strains appeared to be similarly neurotropic as comparable CNS changes and viral loads were found with the two strains, one isolated in Nantes, the other in San Francisco, and with a third isolate in Lyon (results not shown). Different mechanisms which are not mutually exclusive have been proposed (Nottet et al, 1995) to explain FIV, SIV or HIV entry in the brain. Expression of adhesion molecules on the brain microvascular endothelial cells, either directly induced by the infected monocytes, or by the acute immune reactivity generated during primary infection, allowing binding and infiltration of infected monocytes is certainly one of the routes of brain infection involved during early stages via a 'Trojan horse' mechanism (Sasserville et al, 1992). Early neuropathological changes such as gliosis and in situ spots characterized in or around vessels at 7 days p.i. (Figure 2a) as described in rhesus SIV model (Chakrabarti et al, 1991) provide direct evidence for this hypothesis. Nevertheless perivascular brain macrophages resulting from turnover of microglia from bone marrow precursors as described in rodent (Hickey and Kumura, 1988) could also represent a physiological process involved in brain infection, as perivascular macrophages are found in CNS of normal individual (Esiri and McGee 1986).

During the asymptomatic stage at 2, 6 and 12 months p.i., the infection is limited as few infected cells are found associated with gliosis, glial nodules and sometimes WPM. These CNS abnormalities are similar to those described following experimental inoculation of SIV in the monkey (Ringler et al, 1988; Sharer et al, 1988; Chakrabarti et al, 1991; Hurtrel et al, 1993). Nevertheless, compared to monkeys, the lesions in the cat progress less rapidly to severe encephalitis. This is due to shorter course in SIV model in which the late stage appears between 3 months to 3 years after infection (Ringler et al, 1988), whereas in the cat this stage generally does not arise before 6 years (Yamamoto et al, 1988; Pedersen et al, 1989; Ackley et al, 1990; Novotney et al, 1990; Barlough et al, 1991; Callanan et al, 1992). There are very few neuropathological studies at the early stages of HIV-infection, comparable to our experimentally infected cats. The brains of HIV seropositive cases who died from unnatural causes were examined in three reports and compared with these of seronegative cases with similar causes of death (Lenhardt et al, 1988; Gray et al, 1992, 1993). White matter changes including myelin pallor and gliosis were conspicuously more frequent and severe in seropositive cases as described in the FIV model. Perivascular inflammation was significantly more frequent and severe in human in which true vasculitis with infiltration of the vessel wall, lymphocytic meningitis and inflammation of the choroid plexus were observed.

During the asymptomatic period and the terminal phase, in naturally FIV-infected cats, the number of infected cells replicating the virus increased, especially in animal presenting perivascular infiltrates associated with gliosis and glial nodules. Due to the decision of numerous owners to sacrifice the animal immediately after the FIV diagnostic, the immunodeficiency status of animal was estimated with LN analysis during necropsy and histopathological examinations. Classically in FIV infected cat, as described in HIV and SIV infections, immunodeficiency is associated with hypoplasia and preAIDS stage with marked hyperplasia (Yamamoto et al, 1988; Pedersen et al, 1989; Ackley et al, 1990; Novotney et al, 1990; Barlough et al, 1991; Callanan et al, 1992). Cats with the higher viral load in brain presented numerous associated pathologies including lymphadenopathy characterized by a severe hypoplasia or hyperplasia. On the other hand, cats often young, presenting slight syndrome with LN hyperplasia or slight hypoplasia were sacrificed during asymptomatic period and the number of infected cells replicating virus in brain were comparable to those found in experimentally infected animals at 6 or 12 months p.i.

The brain lesions found in the naturally infected group included diffuse gliosis, glial nodules, occasional perivascular infiltrates, WMP, vascular calcification and meningitis, and were more severe than those observed during early stages of infection. They were markedly different from those found in human or monkey in the terminal stages of infection. We did not find the multinucleated giant cells encephalitis described in a few percentage of individuals and characteristic of HIV encephalitis in man and macaque (Budka, 1986; Ringler et al, 1988; Sharer et al, 1988; Chakrabarti et al, 1991; Gray, 1993). We observed such isolated giant cells in one coinfected FIV-FeLV cat, already described in a previous study (Hurtrel et al, 1992). In the present study only cats infected with FIV and sacrificed in optimal conditions, with a short delay between death and the collection of brain parenchyma, were selected. Histopathological analysis of the brain was performed on numerous other cats, not presented here, confirming that CNS lesions characterized in the selected cats used for the quantification of viral load are representative of the FIV encephalopathies. In man, WMP on myelin stains, occasional microglial nodules, mineralization of the vessel walls, and the absence of multinucleated giant cells can be observed in ARC and HIV-1 related dementia (McArthur et al, 1989) and HIV replication measured by viral gene expression is not always correlated with neurological impairments (Kure et al, 1990; Dickson et al, 1991; Gendelman et al, 1994; Wiley and Achim, 1994). Mean number of FIV infected cells, expressed by 50 mm^2 of surface area, in brains

varied from one to eight infected cells per 50 mm^2 (Figure 4) and were comparable to those found at comparable stage in the SIV monkey model. Nevertheless, in the SIV model, animals presenting severe immunodeficiency had a much higher number of infected cells which might be associated with multinucleated giant cells encephalitis as described in human (Chakrabarti et al, 1991).

These differences could be due to the lack of two HIV proteins, Vpr and Nef in FIV whereas the receptor of FIV seems to be the homologous of human CD9 (Hosie et al, 1993; Willett et al, 1994). This could explain both the lower pathogenesis and the larger tropism of FIV which infects T4, T8 lymphocytes, macrophages and B cells (Brown et al, 1991; English et al, 1993; Norimine et al, 1993; Willett et al, 1993; Beebe et al, 1994). It is now clearly demonstrated that the target cell for HIV-1 in the brain is the macrophage and that FIV replicates well in monocytes and macrophages (Brunner and Pedersen, 1989; Beebe et al, 1994). Thus a majority of the cell replicating FIV detected in the brain are probably cells originating from monocytes. Nevertheless, we could not exclude some replication of FIV in astrocytes as described in vitro (Dow et al, 1992). Indeed the low number of infected brain cells in cats together with the difficulty to perform combined immunohistochemistry and in situ hybridization (as antibodies generally used in human or mice to characterize the infected cells do not cross-react with cat antigens) did not allow us to precise the nature of the FIV brain target cells in vivo. Some correlation between viral load in LN and brain was found during the early stages of infection. Moreover, corroborating the implication of the acute immune response in the entry of infected cells in brain, animals analyzed at 7 days p.i. showed a dissociation between the two events with a higher number of infected cells detected in the brain. In late stages, a better correlation between viremia in brain and LN was found, except in animal 431 presenting a drastic LN hypoplasia. Nevertheless, it must be noted that the viral load in the brain is always low compared to the high viral burden in lymphoid tissues.

In conclusion, the low number of virus replicating cells characterized in brain contrasts with the severity of lesions generated and neurological dysfunctions described such as deficits in central sensory, peripheral sensory, motor conduction velocity measures and sleep architecture abnormalities similar to the effects of HIV on the human CNS (Pedersen et al, 1987; Dow et al, 1990, 1992; Hurtrel et al, 1992; Podell et al, 1993; Phillips et al, 1994; Prospero-Garcia et al, 1994; Darko et al, 1995). These findings are consistent with the hypotheses suggesting that brain injuries are mediated in these infections via diffusive factors and amplification processes through cytokine cascades and cell activations.

Materials and methods

Animals, inoculation

Early brain changes were studied in 11 cats specific pathogen free, obtained from IFFA-CREDO (Domaine des Oncins, St Germain sur l'Arbresle, France). Animals housed in isolated rooms in the Veterinary School of Nantes were infected at 6-months-old by IV injection of viral supernatant containing 110 000 RT units. Cats were injected with two different isolates: five with the FIV-Petaluma strain, provided by Dr NC Pedersen (University of California, Davis, USA), and six with the FIV-Envnip strain, isolated in an FIV infected animal. They were sacrificed (one animal at each time point) at 7 and 15 days, and at 1, 2, 6 and 12 months post inoculation (p.i.). All procedures with animals were performed under anesthesia. To prevent any in vitro adaptation of these viruses, the two isolates were inoculated in two SPF cats and then grown during the primary infection in primary peripheral blood mononuclear cells. Cultures were maintained as previously described (Pedersen et al, 1987) and supernatants were tested for reverse transcriptase (RT) activity and to be free of feline leukemia virus (FeLV) and feline syncytium-forming virus (FeSFV) using reference serums of FeLV and FeSFV, by indirect immunofluorescence antibody test performed on fixed FIV infected cells. Two virus stocks were constituted and stored at −80°C with supernatants producing the higher RT activity. Sera of infected cats were tested for the presence of FIV antibodies, using a commercial enzyme-linked immunosorbent assay (ELISA) (IDEXX Corp., Portland, Maine, USA), and all animals infected were found to be positive after 15 days p.i. Late brain changes were studied in eleven naturally FIV-infected cats, collected from the Veterinary School of Nantes, and which were subsequently euthanized at the request of their owners. For this study animals presenting viral coinfections with FeLV or feline infectious peritonitis virus (FIPV) were discarded. The population of cats selected, infected with FIV only, presented the classical clinical signs of FIV infection, including chronic renal insufficiency and chronic digestive diseases (Table 2).

Histopathology

Complete necropsy and histopathological examinations were performed, and a systematic study was carried out on the brain. For each animal, one cerebral hemisphere, half of the cerebellum and half of the brainstem were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin. Histopathological analysis was performed on seven different brain sections: A30, A20, A10, AO, P5 as defined by the stereotaxic atlas (Snider and Niemer, 1961), cerebellum and medulla oblongata. Findings in infected cats were compared

with those in uninfected cats. *In situ* hybridization was performed on the other half, cooled in liquid nitrogen and stored at −80°C. For all used techniques, infected cats were compared to three uninfected controls.

Preparation of ^{35}S-labeled RNA probe and in situ *hybridization*

RNA probe was derived from the transcription vector Bluescript (Stratagene) in which a fragment of the FIV 34TF10 plasmid clone, kindly provided by Dr JH Elder (Research Foundation of Scripps Clinic, La Jolla, USA) spanning the *pol* gene (nucleotides 5481 to 3980) was inserted (Talbott *et al*, 1989). The antisense probe FIV aPpol used to detect FIV RNA was generated from the T7 promoter by *in vitro* transcription of 1 μg of plasmid template with 50 units of T7 RNA polymerase in the presence of 50 μCi of ^{35}S UTP and ^{35}S ATP. Two labeled nucleotides were included in the transcription reaction so as to increase the specific activity of the probe. After incubation for 1 h at 37°C, the DNA template was digested with 10 units DNaseI for 15 min at 37°C. To enhance the penetration of the probe into tissue sections, the ^{35}S-labeled RNA was subjected to mild alkaline hydrolysis in NaHCO₃ 80 mM and Na₂CO₃ 120 mM at 60°C. The hydrolysis time was optimized to obtain a majority of fragments in the 150−200 nucleotides range. After neutralization with Na acetate 600 mM and acetic acid 167 mM, the probe was purified by phenol chloroform extraction and ethanol precipitation. Specific activity ranged between 5.10^7 and 10^8 dpm/μg of input DNA. Hybridization techniques, based upon published procedures (Singer *et al*, 1987), were carried out on tissue sections recently cut on the cryostat, to minimize RNA degradation during storage. Sections were fixed in 4% paraformaldehyde and acetylated in acetic anhydride/triethanolamine pH8 to minimize background. Sections were denatured in 70% formamide at 70°C for 2 min to enhance the accessibility of nucleic acids. The hybridization mix contained the ^{35}S riboprobe at 50 000 dpm/μl, 50% formamide, 10% w/v dextran sulfate, 0.3 M NaCl, 20 mM Tris pH7.5, 5 mM EDTA, 10 mM NaH₂PO₄, 1 × Denhardt's, 0.5 mg/ml yeast tRNA and 100 mM

dithiothreitol (DTT). The mix was heated at 80°C for 2 min and applied to slides. Coverslips were mounted and sealed with rubbercement, and hybridization was carried out at 45°C overnight in a humid chamber. Slides were rinsed successively in 2 × SSC 10 mM DTT for 1 h at room temperature (r.t.), in 50% formamide 2 × SSC 10 mM DTT for 20 min at 60°C, in 2 × SSC for 1 h at r.t., in 0.1 × SSC for 30 min at r.t., then dehydrated in ethanol with 0.3 M ammonium acetate. Slides were coated with NTB2 nuclear track emulsion (Kodak) diluted 1 : 1 with 0.6 M ammonium acetate, and autoradiographed for 28 days at 4°C. The long exposure time ensured that weak hybridization signals were detected. After exposure, slides were treated with Kodak D-19 developer, fixed, and stained with hematoxylin-eosin. The same hybridization processing was performed on positive controls: FIV infected cultured lymphocytes spotted onto slides coated with 3-aminopropyltriethoxysilane (Sigma), and negative controls: brain tissue from uninfected controls. Infected cells were counted on a minimum of ten complete sections performed in frozen blocks harvested between A20 and A25 for the anterior region and between A10 and A15 for the median region as defined by the stereotaxic atlas (Snider and Niemer, 1961). Results are expressed as a mean by 50 mm². With the same procedure productively infected cells were counted in LN. The means obtained for a minimum of four slides were expressed by 5 mm² area. To minimize experimental variability in *in situ* hybridization assay, in each *in situ* experiment there is one section of all animals analyzed, two positive controls and two non infected animal tissue sections.

Acknowledgements

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