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Ramified feline microglia selects for distinct variants of feline immunodeficiency virus during early central nervous system infection

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It is widely accepted that human immunodeficiency virus (HIV) invades the central nervous system (CNS) shortly after peripheral infection to establish a persistent infection of tissue-resident microglial cells. To what extent this early CNS infection is of pathogenic relevance is a matter of discussion. It is conceivable, however, that infected microglia releases virus variants of enhanced neurotropism and/or neurovirulence compared to peripheral isolates. Moreover, microglial variants may exhibit high resistance to antiviral therapeutics that poorly penetrate into brain tissue. The molecular basis of these biological properties is suspected to be associated with specific sequences in the viral *env* gene, particularly within the V3 loop. Therefore, we analyzed in the animal model of feline immunodeficiency virus (FIV) infection of cats lentiviral V3 sequences in highly purified microglial cells and blood from acutely infected animals. Compared to the inoculated virus, nucleotide sequence alterations in serum samples were rarely detectable, if at all. In contrast, up to 19 nucleotide exchanges could be identified within FIV V3 from microglia, resulting in a mutation frequency of up to 14.5% with respect to the deduced amino acid sequence. These findings suggest selection of specific virus variants by brain-resident target cells that might have implications for antiretroviral drug design. *Journal of NeuroVirology* (2003) **9,** 465–476.

Keywords: encephalopathy; FIV; lentivirus; microglia

Introduction

HIV infection of man is regularly complicated by lentiviral invasion of the central nervous system (CNS), resulting in multiple neurological complications (Heaton *et al*, 1994; Janssen *et al*, 1991; Navia *et al*, 1986; Snider *et al*, 1983). Although recent progress in antiretroviral therapy has strikingly reduced frequency of the most severe manifestations (Sacktor *et al*, 2001), neurobehavioral abnormalities remain important clinical features of human immunodeficiency virus (HIV) infection (Sacktor *et al*, 2002). This holds true not only for end-stage diseased persons but also for pre–acquired immunodeficiency syndrome (AIDS) patients who are affected by an increasing incidence of subtle neuropsychological impairments (Albert *et al*, 1995; Marcotte *et al*, 1999). Moreover, mortality of HIV-positive neuropsychologically diseased subjects is significantly elevated compared to asymptomatic individuals (Ellis *et al*, 1997). These clinical observations expand data from virologic and pathologic studies (An *et al*, 1999; Grant *et al*, 1987; Gray *et al*, 1996), suggesting that HIV usually invades the CNS during primary viremia to establish a persistent infection of brain cells. For what

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reasons, however, severity of the resulting neurological symptomatology varies widely from patient to patient is not yet clear. To explain this diversity, several hypotheses have been postulated, including variable production of neurotoxic substances by infected brain cells (Dreyer *et al*, 1990; Giulian *et al*, 1990; Pulliam *et al*, 1991; Sabatier *et al*, 1991), as well as emergence of distinct neurovirulent HIV strains. Indeed, differences in HIV envelope sequences, particularly affecting the hypervariable region V3, from the CNS of asymptomatic and demented patients have been reported (Power *et al*, 1994) (Korber *et al*, 1994; Kuiken *et al*, 1995). This is of special interest because the V3 domain is well known to elicit neutralizing antibodies (Javaherian *et al*, 1990; Rusche *et al*, 1988) and to determine viral cell tropism (Hwang *et al*, 1991). Functional studies revealed that brain-derived retroviruses are preferentially macrophage-tropic (Gorry *et al*, 2001; Smit *et al*, 2001) and recombinant HIV clones containing V3 sequences from the CNS of neurologically impaired individuals induce neuronal apoptosis more efficiently than do V3 loops from clinically inapparent patients (Ohagen *et al*, 1999; Power *et al*, 1998b). Although these findings suggest existence of brain-specific virus variants, data have to be interpreted carefully because they were collected using crude brain homogenate or cerebrospinal fluid (CSF) specimen that may not appropriately reflect the biologically relevant viral population in brain-resident cells. In addition, only a limited number of patients were investigated and samples were usually taken at autopsy when the degrading blood-brain barrier allows unlimited access of peripheral virus strains to the CNS (Boven *et al*, 2000; Persidsky *et al*, 2000; Power *et al*, 1993).

In light of these experimental drawbacks, it is clear that animal models are required to study the dynamics of retroviral evolution in periphery and brain tissue during the early phase of the infection. Feline immunodeficiency virus (FIV) infection of cats has been shown to be a valuable animal model of human AIDS (Pedersen *et al*, 1987; Willett *et al*, 1997a). FIV-infected animals develop a disease syndrome, including neurological sequelae, that closely resembles that of HIV-infected individuals (Podell *et al*, 2000). Similar to HIV, the FIV envelope glycoprotein consists of several conserved and variable regions (Pancino *et al*, 1993; Phillips *et al*, 1990). Among the latter, the V3 domain contains immunodominant sites that induce neutralizing antibodies (de Ronde *et al*, 1994; Lombardi *et al*, 1993) as well as cytotoxic T-cell responses (Flynn *et al*, 1995). Moreover, FIV V3 has been found to be of key importance in viral cell tropism (Siebelink *et al*, 1995; Vahlenkamp *et al*, 1999; Verschoor *et al*, 1995). Recent reports indicate that replication efficiency of FIV in brain-resident cells (Billaud *et al*, 2000) and neurotoxicity (Gruol *et al*, 1998; Johnston *et al*, 2002; Power *et al*, 1998a) is dependent on specific regions within the envelope gene. To study the pathogenic role of these domains in more detail, we aimed at genotyping the V3 domain of FIV strains from periphery and ramified microglial cells, the major lentiviral targets in the CNS (Dow *et al*, 1992; Hein *et al*, 2001). For the first time, we present a detailed sequence analysis of lentiviral variants isolated directly from brain-resident cells of acutely infected animals. Our data suggest that ramified microglia is infected as early as 14 days post infection (dpi) and selects for distinct FIV strains. These might form a reservoir of neurovirulent and/or drug-resistant viruses capable to reseed peripheral organs.

Results

V3 variants in blood and microglia

Ramified microglial cells were previously shown by our group to serve as lentiviral targets in the brain of adult FIV-infected animals, even though during the latent phase of the infection the percentage of FIV producing microglia in CNS tissue is less than 1% (Hein *et al*, 2000). Nevertheless, there is viral replication in the brain and therefore it is feasible that viruses of distinct neurovirulence and/or neurotropism are released at the early stage of the disease. To get an overview about composition of the CNS-associated virus population during the acute phase of the infection, we analyzed the highly variable V3 region of the FIV envelope glycoprotein gp100. Six cats were inoculated intravenously with 5×10^3 cat infectious $doses_{50}$ (CID_{50}) of FIV Wo and sacrificed 14, 28, 92, and 183 days later or left untreated as controls. CNS-located leukocytes were isolated by percoll gradient centrifugation and purity of microglia was determined first by triple-immunolabeling for the β_2 integrine CD18, the leukocyte common antigen CD45, and major histocompatibility class (MHC) II antigens and flow cytometry. $CD45^{low}$-expressing cells, previously defined as resident microglial cells (Sedgwick *et al*, 1991), typically amounted to more than 90% of the total brain-extractable leukocyte population. In contrast, the proportion of blood-derived leukocytes expressing high levels of CD45 never exceeded 1% of the CNS-associated hematopoetic cell fraction (Figure 1). Subsequently, paired samples of microglial cells and serum from individual animals were subjected to reverse transcription (RT)-nested polymerase chain reaction (PCR) specific for FIV V3. PCR products were denatured and then electrophoresed for single stranded conformational polymorphism (SSCP) analysis on standard nondenaturing polyacrylamide gels. Running conditions were chosen so that the two strands of the PCR products migrated with different mobilities according to their sequence-based three-dimensional conformation. Part of the strands, moreover, reassociated to one or several DNA hybrids depending on complementarity of the individual PCR strands. By that means, highly variable FIV V3 sequences could be detected in microglial but not in blood specimen

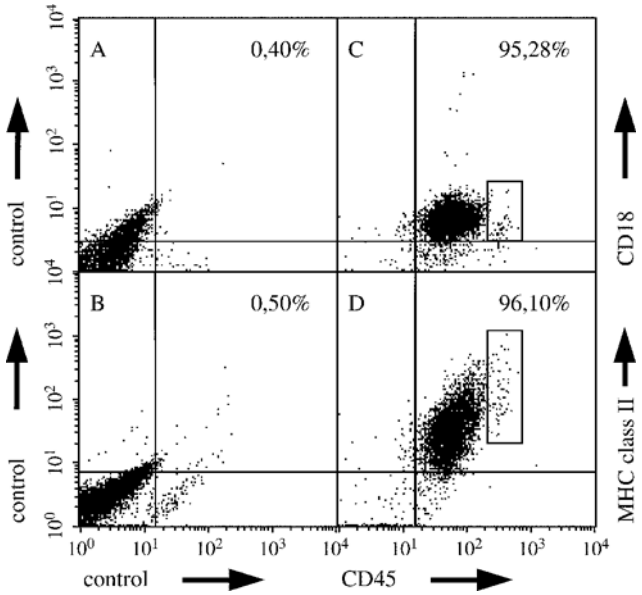

Figure 1 Representative cytofluorographical analysis of adult feline microglia. CNS cells were triple-labeled either with irrelevant isotype-matched control antibodies (**A, B**) or monoclonal antibodies specific for CD18, CD45, and MHC class II (**C, D**) and assessed on a flow cytometer. Boxes in **C** and **D** highlight the population of infiltrating leukocytes. Numbers in the upper right quadrants indicate the percentages of double-positive cells.

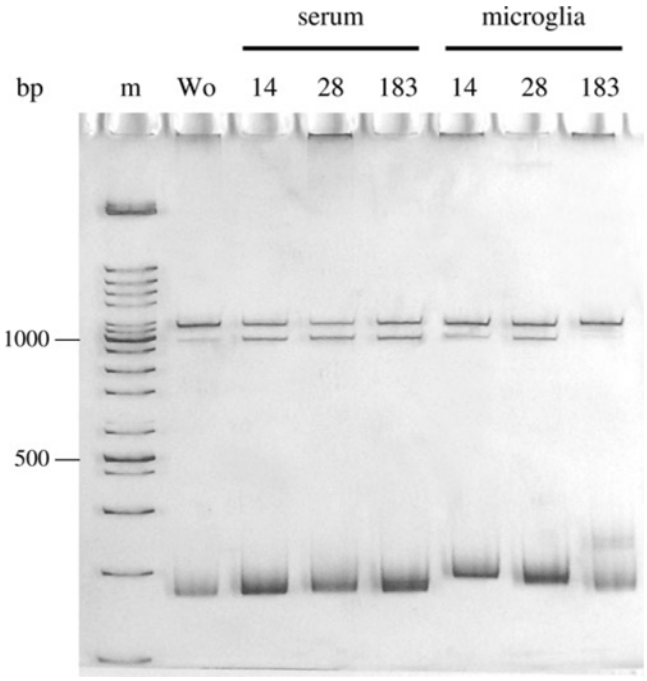

Figure 2 SSCP analysis of FIV V3 amplicons from various tissues of infected animals. At different time points post infection, total RNA from serum and microglia of cats 377 (14 dpi), 653 (28 dpi), and 384 (183 dpi) was extracted and subjected to reverse transcription and FIV V3–specific amplification. Following denaturation, PCR products were electrophoretically separated on nondenaturing polyacrylamide gels and silver stained. Note additional single strand and heteroduplex in microglial V3 from cat 384 (183 dpi). bp, base pairs; m, DNA molecular size marker; Wo, FIV Wo.

(Figure 2). Whereas serum-derived virus isolates behaved similarly in electrophoretic mobilities compared to the inoculated FIV Wo strain, FIV V3 amplicons from microglial cells displayed a completely different SSCP pattern. Mobility shifts were most prominent with respect to DNA hybrids and registerable as early as 14 dpi. Also, relative intensities as well as number of single PCR strands and DNA hybrids varied throughout the whole time of the infection (Figure 2).

Sequencing of V3 regions

Because SSCP data suggested differences in composition of FIV envelope V3 regions, we decided to investigate potential sequence variabilities between microglial and peripheral FIV strains in more detail. The same PCR products used for SSCP analysis were cloned and at least 10 serum- and microglia-derived recombinants, respectively, were analyzed by sequencing. Eventually, individual clone sequences were pooled to design consensus nucleotide sequences of 186 bp, summarized in Figure 3. Compared to the inoculum virus, V3 loop–specific variations in FIV-contaminated serum samples were rarely detectable and limited to the neutralization epitope (Lombardi *et al*, 1995), spanning from nucleotide positions 7418 to 7483 with respect to the FIV Petaluma *env* gene (Talbott *et al*, 1989). Three out of six animals displayed only a single point mutation and serum FIV V3 from one cat even exhibited 100% identity with the inoculated Wo strain. In contrast, microglia-derived FIV isolates showed numerous nucleotide exchanges, not only within the neutralization epitope but also scattered throughout the total V3 region. This holds true for all animals except cat 656 whose microglial isolate differed from the inoculum at only one site. Mean mutation frequencies in FIV V3 from microglia reached up to 18 nucleotide exchanges (9.7%) at 14 dpi, to decline to 10 nucleotide changes (5.4%) at 183 dpi. In serum, however, sequence divergences referring to the inoculated FIV strain never exceeded 1.6%, corresponding to 3 basepair substitutions (Figure 3 and Table 2).

To estimate the consequences of nucleotide exchanges onto the corresponding translation product, the deduced amino acid sequences from the various V3 loop amplicons were analyzed (Figure 4). It is of note that both, serum, and microglial samples

Table 1 Designation of cats and time past FIV infection at necropsy

Animal no.	Days postinoculation
377	14
382	14
653	28
656	92
378	183
384	183

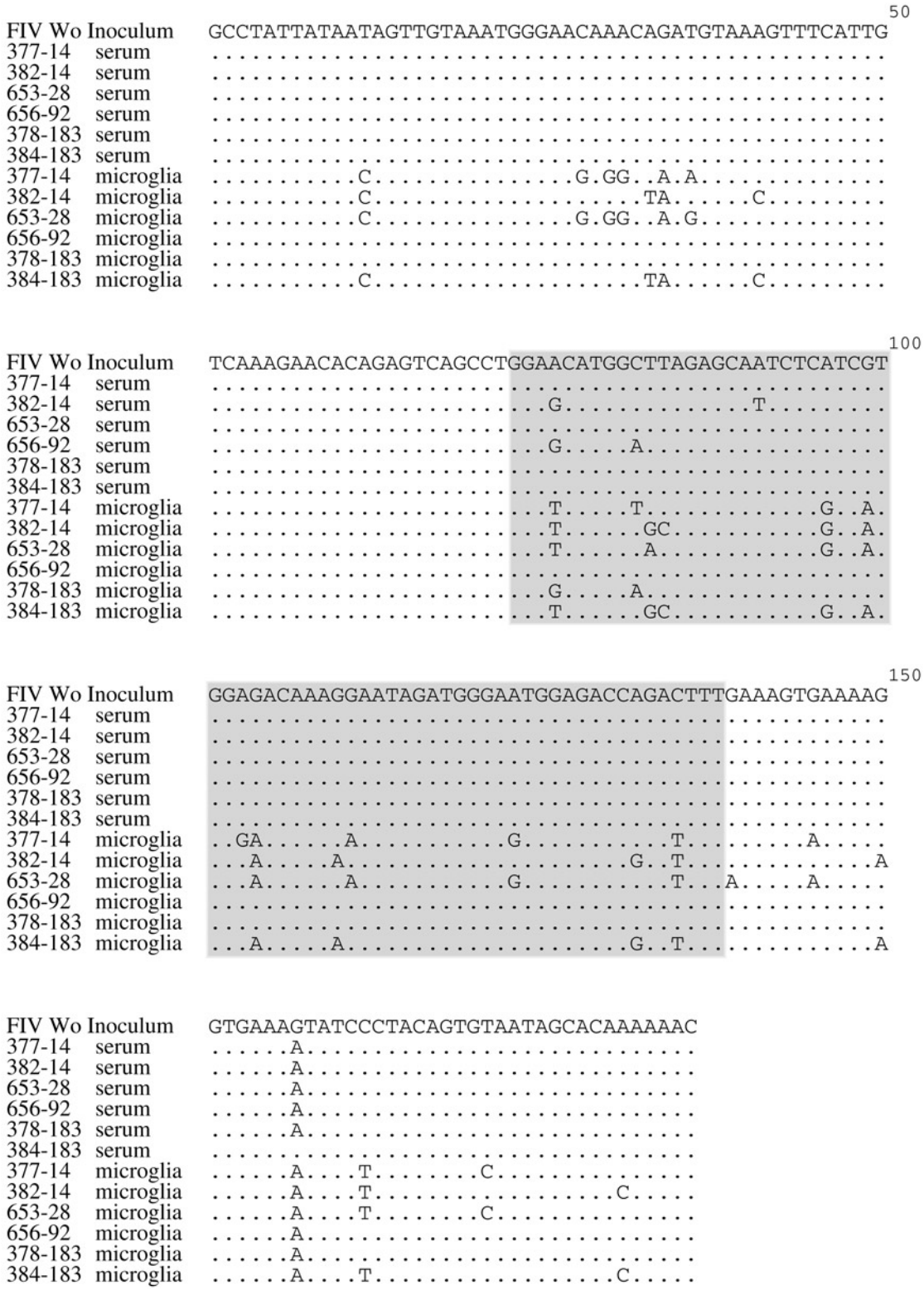

Figure 3 Nucleotide sequence comparison of the FIV envelope V3 loop from paired serum and microglial samples of infected animals. At 14, 28, 92, and 183 days post infection, respectively, FIV V3–specific RNA sequences were reverse transcribed, amplified, and cloned. At least 10 recombinants were sequenced and consensus sequences thereof are presented. The neutralization epitope is colored in gray.

Table 2 Mutation frequencies in V3 nucleotide sequences from FIV-infected cats

Animal no.	Days postinoculation	Tissue	Mutation frequency V3 [%]
377	14	Serum	0.5
382	14	Serum	1.6
653	28	Serum	0.5
656	92	Serum	1.6
378	183	Serum	0.5
384	183	Serum	0.0
377	14	Microglia	10.2
382	14	Microglia	9.1
653	28	Microglia	10.2
656	92	Microglia	0.5
378	183	Microglia	1.6
384	183	Microglia	9.1

displayed a common amino acid change from valine to isoleucine (V → I) at amino acid position 413. Aside, one third of serum samples were characterized by two additional amino acid changes (3.2%), each corresponding to number and distribution of point mutations at the nucleotide level. In comparison, amino acid mutations could be identified in all microglia-derived FIV V3 fragments, and frequency of sequence alterations ranged from a single amino acid shift (1.6%) in cat 656 to nine amino acid exchanges (14.5%) in cat 653. Modifications were not limited to the neutralization epitope as in the case of peripheral isolates, but also tend to cluster upstream of the neutralization epitope at amino acid positions 370 to 374. Here, in two of six samples one potential *N*-linked glycosylation site was introduced by an aspartic acid to asparagine (D → N) substitution at amino acid position 372. Moreover, the majority of microglial variants revealed a remarkable accumulation of amino acid modifications at sites that are suspected to play a role in FIV tropism (Vahlenkamp *et al*, 1999). These include changes of arginine to glutamic acid or lysine (R → E/K) at amino acid position 395, of arginine to lysine (R → K) at position 397, of glutamic acid to lysine (E → K) at position 409, and of valine to isoleucine (V → I) at position 413 (Figure 4).

With respect to the high frequency of mutations in V3, it was reasonable to assume that microglial cells select for distinct FIV variants. To test this hypothesis, we reconstructed evolutionary relationships among V3-specific nucleotide sequences. This phylogeny was estimated using the maximum-likelihood method combined with the neighbor-joining algorithm and the Tamura-Nei model of substitution (Tamura and Nei, 1993). Confidence in the reconstruction of various clades was determined by 1000 replications of the bootstrap procedure. Under these statistical conditions, V3 sequences from serum and microglia fall within distant clades, except for two microglial V3 derived from cats 656 and 378 that cluster with the serum strains. Each monophylogenetic group is strongly supported with a bootstrap value between 92% and 98% (Figure 5). Neighbor-joining phylogenetic analysis based on the deduced amino acid sequences gave a similar tree (data not shown). We also calculated genetic distances to quantify the extent of divergences. For clade 1 comprising mainly serum strains, divergence averaged 1% related to FIV Wo. In contrast, clade 2, exclusively

Cat	Days post inoculation	Tissue	Amino acid sequence V3 (370 380 390 400 410 420)
	0	serum	AYYNSCKWEQTDVKFHCQRTQSQP**GTWLRAISSWRQRNRWEWRPDF**ESEKVKVSLQCNSTKN
377	14	serum	..... I .....
382	14	serum	**A....F**..... I .....
653	28	serum	..... I .....
656	92	serum	**A.I**..... I .....
378	183	serum	..... I .....
384	183	serum	.....
377	14	microglia	.....EAK.....**S.F.....E**..... I .....
382	14	microglia	.....N.T.....**S.R.....K.K**..... I .....Q.
653	28	microglia	.....EAK.....**S.H.....K**.....K.K...I .....
656	92	microglia	..... I .....
378	183	microglia	**A.I**..... I .....
384	183	microglia	.....N.T.....**S.R.....K.K**..... I .....Q.

Figure 4 Comparison of deduced amino acid sequences of FIV V3 from different tissues of infected cats. Based on consensus nucleotide sequences, V3-specific amino acid sequences were predicted using the DNASIS sequence analysis software. Amino acid positions are indicated according to the published FIV Wo envelope sequence. Bold letters mark the neutralization epitope and gray boxes highlight residues presumably involved in FIV tropism.

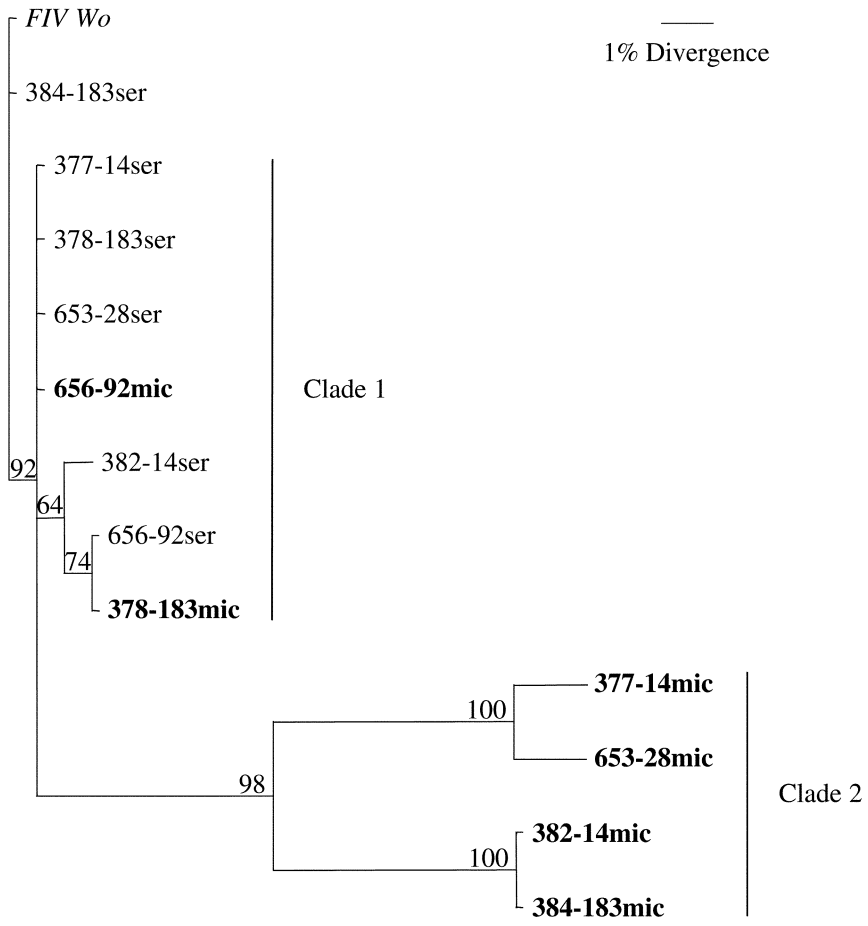

Figure 5 Phylogenetic reconstruction of V3-specific nucleotide sequences from serum and microglia of FIV-infected animals. Each sequence is designated by the cat number, time point post infection, and tissue origin. Branch lengths are shown in proportion to divergence of sequences. Bootstrap values indicate the relative amount of support for the indicated clades as the number of times that clade was recovered in 1000 pseudoreplications.

formed by microglial viruses, diverged 9.7% from the inoculum. These findings clearly indicate that there is clustering of *env* V3 sequences in FIV-infected microglial cells.

Discussion

Since the first reports of AIDS-associated neurological complications were published in 1983 (Snider *et al*, 1983; Whelan *et al*, 1983), extensive investigative efforts were made to elucidate the complex nature of HIV-induced CNS disease. Nevertheless, the mechanisms underlying the clinical and pathological manifestations of central nervous HIV infection are still enigmatic. It is generally agreed, however, that HIV invades the brain early in the course of systemic infection to remain in CNS tissue throughout patient's lifetime. Pathogenic consequences of this lifelong persistence might be manifold. For example, because of poor drug penetration into the CNS, infected brain cells may act as a reservoir for drug-resistant viruses that might reseed peripheral organs of antiretrovirally treated individuals. Further, specific neurovirulent virus strains may arise, inducing pathological alterations and neurocognitive impairments. In support of these ideas, phylogenetic and sequence comparisons of HIV *env* V3 sequences derived from brain and peripheral organs imply compartimentalization of virus in CNS tissue (Chen *et al*, 2000; Gatanaga *et al*, 1999; Korber *et al*, 1994; Smit *et al*, 2001). Data accumulated so far, however, are mainly based on tissue specimen unsuitable to characterize the viral population in CNS-resident cells. In contrast, we used in the current study highly purified microglial cells from the brain of FIV-infected cats to examine potential selection of specific neuroinvasive virus strains.

SSCP as well as sequence analyses revealed striking differences within the *env* V3 regions of primary microglia-derived and peripheral FIV isolates. Whereas V3 sequences of inoculum and serum FIV were nearly identical, microglial V3 displayed numerous mutations at both the nucleotide and the

amino acid levels, particularly within the neutralization epitope. Interestingly, amino acid substitutions were present at positions that have been demonstrated to be critical for FIV tropism (Vahlenkamp *et al*, 1999). Compared to peripheral isolates characterized by V3 sequences typical of lymphotropism, microglial V3 suggest a more macrophage-tropic phenotype. This is in line with previous data indicating that HIV isolates from the brain infect preferentially macrophages *in vitro* (Cheng-Mayer *et al*, 1989; Ghorpade *et al*, 1998a) and macrophage-tropism for its part is the major determinant in predicting ability of primary HIV strains to replicate in microglial cells (Gorry *et al*, 2001). Moreover, in the SIV model system, brain as well as gut or lung-derived virus variants not only were macrophage-tropic but also displayed similar nucleotide substitutions within the *env* gene (Desrosiers *et al*, 1991; Kodama *et al*, 1993; Mori *et al*, 1992). Although these findings suggest an overlap of macrophage- and microglial-tropism, brain isolates vary in their replication efficiency in microglia compared to peripheral macrophages (Gorry *et al*, 2001). Also, sequential passage of primary HIV isolates, but not macrophage-tropic clones, through microglial cells results in viral adaptation that is accompanied by distinct amino acid changes within the proviral *env* V3 region (Strizki *et al*, 1996). From this, one might speculate that even though conditions for viral entry in peripheral and CNS-resident macrophages might be similar, the intracellular environment may differently influence viral replication, giving rise to viruses of diverse tropism and/or permissiveness. Summerizing this point, our data allow no decision whether V3 determined tropism for peripheral macrophages or brain-resident microglia is indeed an overlapping property of FIV isolates, or if specificity of the virus for these two macrophage types segregates. To answer this question, we aim at the establishment of recombinant FIV virus clones with V3 sequences from microglial and peripheral macrophages, respectively.

Aside from possible conformational alterations of the V3 region due to amino acid substitutions, an aspartic acid to threonine mutation at amino acid position 372 leads to insertion of a potential *N*-glycosylation site. This is of special interest because glycosylation of the HIV V3 loop has been reported to play an important role in viral infectivity and binding of neutralizing antibodies. For example, low content of V3-associated carbohydrates is linked to the preferential use of the coreceptor CXCR4, whereas acquisition of oligosaccharides is accompanied by a switch to CCR5 usage (Li *et al*, 2001; Polzer *et al*, 2001). Enhanced glycosylation also results in increased resistance to V3-directed neutralization (Back *et al*, 1994; Schonning *et al*, 1996). Similarly, Giannecchini and coworkers (1998) recently described that glycosylation of the FIV envelope is involved in antibody-mediated neutralization. Regarding additional *N*-linked glycosylation sites within microglial FIV V3, it is conceivable that not only viral tropism but also sensitivity to neutralization is changed during microglial passage. In this context, it is worth mentioning that insensitivity to neutralization seems to be a common feature of primary CNS-derived virus strains (Cheng-Mayer *et al*, 1989). Furthermore, *in vitro* selection for brain cell tropism is accompanied by viral resistance to neutralization by anti-V3 monoclonal antibodies (McKnight *et al*, 1995). As a consequence, immune escape mutants might emerge and become predominant particularly at end-stage disease when the host's immune system is compromised. Future work is therefore needed to study the sensitivity of microglia-derived virus variants to neutralization by serum antibodies obtained from the same individual.

Regarding frequent nucleotide exchanges in FIV V3 from microglia, one might expect that these result from vigorous viral replication in CNS tissue. However, comparable to CNS infection with other lentiviruses (Clements *et al*, 2002; Gray *et al*, 1996), microglial FIV expression seems to be very low during the asymptomatic phase of the infection (Hein *et al*, 2000). Therefore, we favor the hypothesis that CNS-specific variants might emerge because of virus selection at the stage of target cell entry. Because we did not use a virus clone for intravenous infection of the animals, it is conceivable that a minor fraction of the lymphotropic bulk of viruses expresses a V3 loop appropriate for infection of microglial cells. As a consequence, this minority will accumulate in microglial cells and thus become the major detectable variant in these brain-resident cells. Under these circumstances, extensive mutations of the viral genome must not necessarily occur within infected brain cells to detect V3 variants distinct from those present in the bulk of peripheral viruses.

Isolation of microglial cells was always accompanied by copurification of a low amount of $CD45^{high}$ leukocytes. From this observation, one might argue that the presented CNS-associated V3 sequences were derived from FIV-infected macrophages contaminating the microglial cell population. Even though this cannot be excluded with any certainty, there are some arguments supporting the idea that the source of the FIV sequences described were indeed microglial cells and not macrophages: (1) Due to the isolation procedure, perivascular macrophages remain stuck to the blood vessels, which are completely removed as partially intact fragments. (2) Fluorescence-activated cell scanning (FACS) analysis of the $CD45^{high}$ CNS cell fraction (not shown) revealed that the latter is almost exclusively composed of T and B lymphocytes. If these would contribute to the CNS viral load, the FIV V3 sequences detectable within brain-tissue should exhibit a more lymphotropic character. In comparison, our CNS sequences differ exactly at the sites defining lymphotropism, suggesting the

sequences not to be obtained from infiltrating lymphocytes. (3) Contamination by leukocytes from the vasculature is unlikely because in this case, the number of $CD45^{high}$ cells within the brain-isolated leukocyte population should be significantly increased. Nevertheless, additional experiments using FIV recombinants are needed to prove specificity of the sequences presented.

Our study concentrated on the lentiviral V3 region because of its significance in target cell entry, antiviral immune response, and neuropathogenesis. However, it is possible that we have to extend our investigations to other parts of the viral surface unit, or to the envelope protein as a whole, to evaluate whether there are additional domains influencing the viral phenotype present in CNS tissue. Indeed, HIV isolates with identical V3 sequences can behave differently with respect to tropism and replication capacity and this might be due to variances within the V1/V2 and C4 regions (Boyd *et al*, 1993; Carrillo and Ratner, 1996; Groenink *et al*, 1992; Koito *et al*, 1995). In FIV, amino acid mutations within the transmembrane domain and in the V4 region of the envelope glycoprotein have been shown to be involved in viral tropism (Vahlenkamp *et al*, 1997, 1999). These findings suggest that regulatory elements outside the V3 region might influence V3 loop conformation and thereby interaction between the viral envelope and its cellular receptor. Hence, when interpreting the relevance of specific V3 sequences to lentivirus-induced pathogenesis, it must be taken into account that additional domains of the viral *env* may be involved.

Taken together, our data clearly indicate selection of distinct virus variants by microglial cells, even though we failed to locate a consistent pattern of molecular changes within the FIV V3 region. Two cats, designated 382 (14 dpi) and 384 (183 dpi), however, exhibited identical amino acid transitions in microglial V3 and therefore are good candidates for further investigations on putative microglia-specific viruses. Besides, our phylogenetic analyses unequivocally revealed clustering of microglia-derived viruses apart from peripheral FIV isolates, supporting the idea of lentiviral adaptation to the CNS-resident macrophage population. The mechanisms involved in that process remain to be determined. In that context, cell physiology will be of key importance, e.g., post-translational processing of viral proteins as already mentioned above. Moreover, preentry factors have to be considered. There is evidence that the principal coreceptors of HIV isolated from the brain are CCR3 and CCR5, and to a minor extent CXCR4 (Albright *et al*, 1999; Gabuzda and Wang, 1999; Ghorpade *et al*, 1998b; He *et al*, 1997; Shieh *et al*, 1998). Type and intensity of coreceptor expression may therefore be decisive for lentiviral infection of microglial cells. Comparable to HIV, FIV utilizes chemokine receptors and surface heparans for cell invasion, depending on target cell type and virus strain (de Parseval and Elder, 2001; Johnston and Power, 2002; Richardson *et al*, 1999; Willett *et al*, 1997b). More recently, CXCR4 expression was reported on feline neurons, brain endothelial cells, and astrocytes (Koirala *et al*, 2000; Nakagaki *et al*, 2001), which is in line with preliminary data of our group implying CXCR4-specific mRNA expression in feline microglial cells (data not shown). So far, however, it is not yet clear if chemokine receptors alone are sufficient for FIV infection or if a combination with other cell surface elements is required. Likewise, expression pattern of potential FIV receptors on feline cells is only partially understood. Against this background, we will construct a number of chimeric viruses containing microglial FIV V3 in a $FIV_{Petaluma}$ backbone to elucidate determinants involved in FIV invasion of microglial cells. From these studies, one might expect new insights into the pathogenic role of microglia as viral reservoir and into potential targets for therapeutic intervention.

Materials and methods

Animals

Cats (strain Ico:Fec Eur, Tif) were purchased from IFFA Crédo (Lyon, France) at 6 months of age and housed under specific pathogen–free (SPF) conditions in an air-conditioned animal facility. They were either infected intravenously with cell-free tissue culture supernatant (TCS) containing 72,000 fluorescence units (FU) of RT activity, equivalent to 5000 CID_{50} of FIV strain Wo, or left untreated. At different time points post inoculation (p.i.), cats were necropsied according to the schedule summarized in Table 1. Housing and handling of animals conformed to local rules for care and use of laboratory animals.

Virus

Strain FIV Wo (kind gift of A. Moraillon, École Vétérinaire, Maison d'Alfort, France) was isolated from blood leukocytes of a cat with full-blown immunodeficiency by coculture with peripheral blood mononuclear cells (PBMCs) from healthy donors. Viral stocks were prepared as previously described (Hein *et al*, 2000).

Isolation of microglial cells

Microglial cells were extracted from the CNS of cats by density-gradient centrifugation as published earlier (Hein *et al*, 2000). Briefly, following meninges stripping and mechanical disruption, the resulting brain cell homogenate was enzymatically digested before being transferred to a gradient consisting of two layers of percoll of different density. Following centrifugation for 20 min at $1250 \times g$, myelin debris was removed and cells were overlayered

onto a second multilayer gradient. During subsequent centrifugation, microglial cells sedimented to the 1.072 g/cm^3 interphase from which they were collected.

Cell labeling and cytofluorographic analyses

Monoclonal antibodies (mAbs) used for characterization of microglial cells were specific for CD18 (clone MHM23, mouse immunoglobulin (Ig)G1; fluorescein isothiocyanate conjugate; Dako, Glostrup, Denmark), CD45 (clone 25-2C, mouse IgM; VMRD, Pullman, USA), and MHC class II (clone vpg 3, mouse IgG1; Serotec, Oxford, UK). Isotype-matched control antibodies (clone 15H6, mouse IgG1; clone 11E10, mouse IgM; SBA, Birmingham, USA) were used as negative controls. Primary antibodies were detected by (*R*)-phycoerythrin– or biotin-conjugated F(ab′)$_2$ fragments of goat anti-mouse IgG and IgM heavy chains, respectively (Dianova, Hamburg, Germany). Biotinylated secondary antibodies were visualized by streptavidin-RED670 (GibcoBRL, Eggenstein, Germany). All labeling steps were performed on ice in a total volume of 50 μl. First round labeling was carried out by resuspending microglial cells (2×10^5) in buffer solution containing mAbs that bind to CD45 and MHC class II and 10% heat-inactivated normal cat serum. mAbs were incubated for 20 min and washed; then a mixture of secondary goat anti-mouse Igs was added and incubated for another 20 min. Cells were washed and conjugate-associated free binding sites were saturated by 20% heat-inactivated normal mouse serum before addition of mAb recognizing CD18 together with streptavidin-RED670. Subsequently, cells were washed and 1×10^4 live-gated events were assessed on a FACScan (Becton & Dickinson, Heidelberg, Germany) using CellQuest analysis software.

RT-PCR and SSCP

For SSCP analysis of FIV V3 sequences, total RNA was extracted from paired serum and microglial samples applying the silicagel membrane technology of Qiagen (Hilden, Germany). cDNA was prepared from DNase I-treated RNA by the First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and subjected to nested PCR. According to previously published data (Nishimura *et al*, 1996), outer primers were 5′-GAGTAGATA C(AT)-TGGTT(AG)CAAG-3′ and 5′-CATCC TAATTCTTG CATAGC-3′. The inner primer set consisted of 5′-CAAAATGTGGATGGTG GAA(CT)-3′ and 5′-ACTCTATCCAAGCTCC (CT)GTTAC-3′, corresponding to conserved regions on each side of the V3 domain. Both rounds of amplification were carried out in a total volume of 50 μl containing 10 mM Tris/HCl (pH 8.8), 5 mM $(NH_4)_2SO_4$, 25 mM KCl, 2 mM $MgSO_4$, 200 μM of each dNTP, 20 pmol of each primer, 2 Units *Pwo* polymerase (Stratagene, Amsterdam, The Netherlands), and 5 μl cDNA and first round PCR product, respectively. PCR was run on the RoboCycler Gradient 96 (Stratagene) with denaturing for 2 min 15 s at 94°C, followed by 40 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min 30 s for the outer and 45 s for the inner PCR reaction, with a final extension of 3 min 30 s at 72°C. If not otherwise stated, PCR reagents were obtained from Roche Diagnostics.

Four microliters (25 ng) of PCR product were mixed with 4 μl of 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, heated at 100°C for 3 min before being snap-chilled on ice for 5 min. Denatured samples were loaded onto a 8% polyacrylamide gel containing 90 mM Tris-borate, pH 8.5, and 2 mM EDTA. Electrophoresis was performed at room temperature with 10 mA for 115 min. Bands were visualized by silver staining according to a method previously described by Axton and colleagues (1997).

DNA sequencing and analysis

V3-specific RT-PCR products were blunt end–ligated into the pCR 4Blunt-TOPO vector (Invitrogen, Carlsbad, USA) following the manufacturer's recommendations and bacterial transformation was done by chemical treatment. Recombinant clones were screened by PCR using the V3-specific inner primer set and sequences of positive clones were determined by automated dideoxy sequencing on an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, USA). Sequence fragments were aligned with MacDNASIS (Hitachi, San Bruno, USA) and phylogenetic tree construction was performed by the maximum-likelihood method, combined with the neighbor-joining algorithm and the Tamura-Nei model of substitution (Tamura and Nei, 1993), using the PUZZLE software package, version 4.0.2. To display phylogenies, the Treeview program (Page, 1996) was applied.

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