Envelope gene sequences encoding variable regions 3 and 4 are involved in macrophage tropism of feline immunodeficiency virus

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The envelope is of cardinal importance for the entry of feline immunodeficiency virus (FIV) into its host cells, which consist of cells of the immune system including macrophages. To characterize the envelope glycoprotein determinants involved in macrophage tropism, chimeric infectious molecular clones were constructed containing envelope gene sequences from isolates that had been propagated in peripheral blood mononuclear cells (PBMC). The progeny virus was examined for growth in PBMC and bone marrow-derived macrophages and viruses with different replication kinetics in macrophages were selected. Envelope-chimeric viruses revealed that nucleotide sequences encoding variable regions 3 and 4 of the surface glycoprotein, SU, are involved in macrophage tropism of FIV. To assess the biological importance of this finding, the phenotypes of envelope proteins of viruses derived from bone marrow, brain, lymph node and PBMC of an experimentally FIV-infected, healthy cat were examined. Since selection during propagation had to be avoided, provirus envelope gene sequences were amplified directly and cloned into an infectious molecular clone of FIV strain Petaluma. The viruses obtained were examined for their replication properties. Of 15 clones tested, 13 clones replicated both in PBMC and macrophages, two (brain-derived clones) replicated in PBMC only and none replicated in Crandell feline kidney cells or astrocytes. These results indicate that dual tropism for PBMC and macrophages is a common feature of FIV variants present in vivo.

Introduction

Feline immunodeficiency virus (FIV) is a lentivirus pathogen of domestic cats. FIV infection is characterized by an impairment of immune functions and a progressive depletion of CD4+ T lymphocytes (Ackley et al., 1990; Pedersen et al., 1987; Siebelink et al., 1990). Pathogenesis of FIV infection is similar to that of human immunodeficiency virus (HIV) infection in man, both leading to AIDS after an extended asymptomatic period (Pedersen et al., 1987; Yamamoto et al., 1988). In addition to CD4+ T cells, the tropism of FIV involves CD8+ T cells, CD21+ B cells, astrocytes, megakaryocytes and monocytes/macrophages (Brunner & Pedersen, 1989; Dow et al., 1992; English et al., 1993; Pedersen et al., 1987). Certain FIV strains that were isolated from diseased cats also replicate in cells of the Crandell feline kidney (CRFK) line (Miyazawa et al., 1989; Nishimura et al., 1996; Phillips et al., 1990; Siebelink et al., 1992; Yamamoto et al., 1988). It is not known whether the phenotype of virus variants present in the asymptomatic phase of FIV infection differs from the phenotype present in the disease phase, as described in about 50% of HIV-1-infected individuals (Tersmette et al., 1989). Differences have been found, however, between the infected cell populations at different times: early after infection, CD4+ T cells are the predominant cellular targets, and the proportion of infected macrophages increases during the acute flu-like illness. In the chronic stage, most infected cells are CD8+ T lymphocytes, B
lymphocytes and macrophages (Beebe et al., 1994; Dean et al., 1996; English et al., 1993).

The envelope glycoprotein plays a key role in the initial virus–cell interaction, although other virus proteins may also be involved. The tropism of FIV for CRFK cells has been shown to be affected by the third variable (V3) region of the surface glycoprotein, SU (Siebelink et al., 1995; Verschoor et al., 1995), and by the ectodomain of the transmembrane (TM) glycoprotein (Vahlenkamp et al., 1997). The V3 region also contains a linear neutralization domain (de Ronde et al., 1994; Lombardi et al., 1993). FIV was shown to use CXCR4 for cell fusion and the V3 region was found to be involved in the interaction (Willett et al., 1997a, b). Although having a different sequence, the V3 region of the viral envelope protein of HIV-1 is also important for tropism, cytopathicity and virus neutralization and is involved in the interaction with CCR5 and CXCR4, the main co-receptors used by the macrophage-tropic and T cell-tropic HIV-1 isolates (Cao et al., 1993; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Levy, 1993; Wain-Hobson, 1996; Wild et al., 1993).

Macrophages play an important role in the immune system as antigen-presenting cells (Beebe et al., 1994; Bendinelli et al., 1995; Levy, 1993) and they are probably also involved in FIV pathogenesis – by harbouring the virus and contributing to its dissemination in the organism. Only a few studies have addressed macrophage tropism (Beebe et al., 1994; Brunner & Pedersen, 1989; Power et al., 1998); FIV was shown to be present in peritoneal macrophages of cats infected with FIV strain Petaluma for 6–18 months. Cultures of peritoneal macrophages were found to be susceptible to infection with this strain, which had been propagated on CRFK cells (Brunner & Pedersen, 1989). By using in situ hybridization, Beebe et al. (1994) showed that the dominant cell type that was infected shifted from T lymphocytes to macrophages during the onset of primary disease. The shift might be due to cytopathic infection of T lymphocytes with subsequent selection of macrophage-tropic variants (Beebe et al., 1994).

In an attempt to identify determinants of macrophage tropism, we focused on the FIV envelope glycoprotein. Chimeric infectious molecular clones derived from peripheral blood mononuclear cells (PBMC)-tropic and macrophage-tropic viruses were constructed and progeny virus was examined for replication kinetics in macrophages and PBMC in vitro. The V3 and V4 regions of the FIV SU glycoprotein were indeed found to possess determinants involved in macrophage tropism. In order to get an impression of the phenotype of virus variants present in a persistently viraemic, experimentally infected healthy cat, we constructed infectious molecular clones by using envelope gene sequences derived directly from tissues of different body compartments. By using this approach, the tropism of variants present in vivo can be determined without introducing possible errors due to in vitro selection. Viruses derived from these clones were tested for their ability to replicate in feline PBMC, bone marrow-derived macrophages, primary astrocytes and CRFK cells. Our results indicate that dual tropism for PBMC and macrophages is a common feature of FIV variants present in vivo.

**Methods**

- **Cell culture.** CRFK-HO6 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal calf serum (FCS). Feline PBMC were maintained in Iscove’s medium supplemented with 200 U/ml recombinant interleukin-2 (Eurocetus). 2.5 μg/ml concanavalin A and 10% FCS. Bone marrow-derived macrophages were prepared as described by Daniel et al. (1993) and cultured in Iscove’s medium supplemented with 15% Hyclone serum and 2 mM glutamine. Primary astrocytes were obtained from newborn kittens, prepared essentially as described by Dow et al. (1992) and tested for glial fibrillary acidic protein positivity prior to infection. All culture media were supplemented with 100 μg/ml streptomycin and 100 IU/ml penicillin.

- **Tissues and virus isolates.** Virus isolates were obtained from the cerebrospinal fluid (CSF) and PBMC of a naturally FIV-infected cat that showed signs of central nervous system involvement by co-cultivation of uninfected feline PBMC. The virus isolates obtained (FIV-UT48), derived from the CSF and PBMC, were stored at −80 °C. Both virus isolates were used to infect feline PBMC and proviral DNA was isolated by using the method described by Boom et al. (1990).

- **Amplification and cloning of envelope gene sequences.** Gene sequences encoding the FIV envelope glycoprotein were amplified by using primers with flanking 5’ MluI (ACGGCGT) and 3’ SalI (GTCGAC) restriction sites (underlined). Primer 217 consisted of the nucleotide sequence 5’ TAGACCGCTAAGATTTTAGATCTC- GATG 3’ (nt 6512–6543; Talbott et al., 1989) and primer 259 consisted of the sequence 5’ CTGTCGACTAAGTCTGAGATACTTCATG- GATG 3’ (nt 5890–5925). PCR was performed for 35 cycles by using the Expand Long Template reaction mixture (Boehringer Mannheim). Each cycle consisted of 1 min at 94 °C, 1 min at 55 °C and 2.5 min at 72 °C. The 50 μl reaction mixture contained 100 ng cellular DNA, 100 ng of each primer, 50 mM Tris-HCl (pH 9.2), 1.75 mM MgCl₂, 14 mM (NH₄)₂SO₄, 200 μM of each deoxynucleoside triphosphate and 0.75 μl of the enzyme mixture. Amplification products were analysed on a 1% agarose gel, purified from the gel by using a QIAquick kit (Qiagen) and digested with the restriction enzymes MluI and SalI. After preparative gel electrophoresis, the purified amplification products were cloned into pPETENV. This vector is basically identical to Pet-14 (Olmsted et al., 1989) but lacks flanking DNA sequences. In addition, pPETENV contains unique MluI and SalI restriction sites to allow selective exchange of envelope gene sequences (Verschoor et al., 1995).

- **Exchange of envelope gene fragments.** To construct envelope-chimeric clones, the MluI–SalI fragments were first cloned into vector pSH, a derivative of pS73 containing MluI and SalI cloning sites (Verschoor et al., 1995). Construction of chimeric clones 27/6 and 8/27 was performed by exchanging the MluI–SalI (1292 bp) and NsiI–SalI (1045 bp) fragments between the parent clones 6 and 27. The resultant chimeric MluI–SalI fragments were subsequently cloned into pPETENV. Sequence analysis confirmed that the fragment exchanges were performed correctly. Exchange of the V3–V4 region was performed.
Macrophage tropism of FIV

Characterization of envelope gene sequences involved in macrophage tropism

Molecular clones containing envelope gene sequences were derived from a cat infected with isolate FIV-UT48. FIV isolates had been obtained after co-cultivation of CSF and PBMC with PBMC from uninfected cats. By using PCR, virus envelope sequences were cloned into an FIV-Petaluma background and the viruses obtained were examined for their replication kinetics in cultures of PBMC and macrophages. For detailed study, we selected clones 8 and 27 from PBMC cultures that did not replicate in CRFK cells. As shown in Fig. 2(a), both clones replicated with similar kinetics in PBMC, virus antigen being detectable in the supernatant from day 4 onwards. However, the replication curves in macrophages were different. In three independent experiments, virus replication of clone 8 could be measured from day 8 onwards, whereas virus antigen in the supernatant of cultures infected with progeny of clone 27 was already detectable on day 4 (Fig. 2b). In addition, the slope of virus production against time was different for the two viruses: clone 8 replicated slightly more slowly than clone 27.

Construction of envelope-chimeric clones

To identify virus determinants responsible for the different phenotypes, we constructed envelope-chimeric clones; a schematic representation of the constructs derived from clones 8 and 27 is shown in Fig. 1. The MluI–NsiI fragment consisted of a part of the envelope leader sequence and encoded the V2,
V3 and V4 regions of the envelope glycoprotein. The Xbal–NsiI gene fragment encoded the V3 and V4 regions of the SU envelope glycoprotein. The results of the infection experiments with progeny of these clones are also summarized in Fig. 1. Virus derived from clone 8/27, with envelope genes consisting of the MluI–NsiI fragment (1293 bp) of clone 8 and the NsiI–SalI fragment (1045 bp) of clone 27, replicated similarly in PBMC and macrophages to virus from the parent clone 8 and the corresponding virus derived from clone 27/8 replicated with similar kinetics to virus from clone 27. Progeny of clone 8/27/8 [MluI–Xbal (602 bp) and NsiI–SalI fragments (1045 bp) derived from clone 8 and Xbal–NsiI fragment (691 bp) derived from clone 27] showed the same replication kinetics as virus from the parent clone, 827. Virus derived from the corresponding clone 27/8/27 showed the same phenotype as virus derived from the parent clone, 8 (Fig. 2). These results indicated that the envelope gene sequence encoding the V3–V4 region is involved in macrophage tropism of FIV.

Sequence analysis of the envelope gene fragment

The MluI–SalI DNA fragments of clone 27 (lymphotropic and macrophage tropic), 8 (lymphotropic with an impaired capacity to replicate in macrophages) and 46 were sequenced and the deduced amino acid sequences were analysed. Clone 46, which was derived from the CSF, was included because progeny virus replicated only in PBMC. Exchange of envelope fragments derived from this clone did not result in replication competent viruses, however. The amino acid sequences encoded by the Xbal–NsiI fragments are shown in Fig. 3. The amino acid changes between clone 27 and clone 8 present within the V3 and V4 regions were lysine to arginine (K→R) at position 395, aspartic acid to glutamic acid (D→E) at position 409, isoleucine to valine (I→V) at position 413, proline to leucine (P→L) at position 423, serine to threonine (S→T) at position 429, asparagine to threonine (N→T) at position 448, and lysine to glutamic acid (K→E) at position 478. When comparing clone 46 with clone 8, two amino acid changes were observed within the V3 region: arginine to lysine (R→K) and glycine to arginine (G→R) at positions 409 and 397, respectively. The P→L change at position 423 resulted in the appearance of a potential N-glycosylation site in clones 8 and 46. The T→N change at position 448, introducing an additional potential N-glycosylation site, was present in both clones 27 and 46.

Cell tropism of infectious molecular clones derived from different cat tissues

Tissue samples were obtained from a cat infected for 6 years with the FIV-UT48 isolate. Fifteen infectious molecular
Table 1. Replication of FIV clones derived from different tissues

Virus replication was determined by measuring p24 production in the culture supernatant by an antigen-capture ELISA. ND, Not done.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Clone</th>
<th>CFRK cells</th>
<th>PBMC</th>
<th>Macrophages</th>
<th>Astrocytes</th>
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<tbody>
<tr>
<td>Bone marrow</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>122</td>
<td>–</td>
<td>+</td>
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<tr>
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<td></td>
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<td>110</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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</table>

Clones were constructed (Table 1). Two clones contained envelope gene sequences derived from the bone marrow (63, 90), three clones were derived from the lymph node (4, 5, 110) and five clones were isolated from each of brain tissue (11, 37, 38, 40, 122) and PBMC (31, 32, 33, 62, 111). An infectious clone containing the envelope gene sequence of FIV-Petaluma (pPet) with MluI and SalI restriction sites was included as a positive control. Except for two clones (37 and 40, which replicated in PBMC only) derived from the brain, all clones replicated in PBMC and macrophages. None of the 15 clones grew in either CRFK cells or astrocytes. The Petauma clone (pPet) replicated in all four cell-culture systems tested.

The V3–V4 envelope gene regions of clones containing envelope genes derived from different tissues of a cat infected for 6 years with FIV-UT48 were sequenced. Analysis revealed a close relatedness of these sequences and clones 27, 8 and 46 of the FIV-UT48 isolate. Differences were too small to show significant bootstrap values. No amino acids or glycosylation sites specific for the exclusively lymphotropic phenotype and present only in the brain-derived clones 37 and 40 (Table 1) and clone 46 could be identified.

Discussion

To characterize virus determinants involved in macrophage tropism, we selected clones with different phenotypes derived from PBMC-propagated FIV isolates: clone 8, which replicates well in PBMC but poorly in macrophages, and clone 27, which replicates well in both cell types. These clones differ monstrably in their glycoprotein gene sequences and the results obtained support the premise that determinants of macrophage tropism are present on the virus envelope. The construction of envelope-chimeric viruses revealed that the V3–V4 region of the SU envelope glycoprotein is important for macrophage tropism. The deduced amino acid sequence obtained from these clones showed that a potential N-glycosylation site at position 448 within the lympho- and macrophage-tropic clone 27 is shifted to position 422 in clone 8.

The exclusively lymphotropic clone 46 contains potential N-glycosylation sites at both positions 422 and 448, suggesting that glycosylation at position 422 does not favour entry into macrophages. In HIV-1, glycosylation in the V3 region is involved in the binding of neutralizing antibodies, is lost upon prolonged culture in a T cell line and is related to the non-syncytium-inducing (NSI) versus syncytium-inducing (SI) character of the virus isolate; all this suggests a role in HIV-1 entry (Back et al., 1994). Our lymphotropic clone, 46, also contained a glycine to arginine mutation at position 397, which adds a positively charged amino acid to the V3 region. Within the V3 region of HIV-1, basic amino acid substitutions or a loss of acidic amino acids are correlated with the T cell-tropic phenotype (Chesebro et al., 1992; De Jong et al., 1992; Fouchier et al., 1992; Shioda et al., 1994; Simmonds et al., 1991). The overall charge of the V3 region in clone 46 may influence the interaction between the V3 region and a cellular receptor.

The V3 region of the SU envelope glycoprotein and the
ectodomain of the TM envelope glycoprotein of FIV have previously been shown to contain virus determinants for CRFK cell tropism (Siebelink et al., 1995; Vahlenkamp et al., 1997; Verschoor et al., 1995). These cell culture-adapted strains of FIV use the chemokine receptor CXCR4 for cell fusion (Willett et al., 1997a, b, 1998; Poeschl & Looney, 1998). The same chemokine receptor is used by T cell line-adapted SI HIV-1 strains, while NSI macrophage-tropic strains make use of the chemokine receptor CCR5 as second receptor (Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). However, the relationship between receptor usage and virus strains seems to be complex, and several dual-tropic strains of HIV-1 have been described (Doranz et al., 1996; Simmons et al., 1996).

Our genotypically and phenotypically characterized FIV clones will be useful in the search for additional receptors and in elucidating the mechanisms involved in the broader cell tropism of FIV. The identification of determinants important for macrophage tropism in the V3 and V4 regions indicates evolutionary links between the basic entry mechanisms of FIV and HIV-1. By comparing virus and cellular determinants involved in cell tropism of FIV and HIV-1, factors responsible for the similar pathogenesis of the two lentivirus infections may be identified.

The limited or absent capacity of clones 8 and 46 to replicate in macrophages could have been the result of their previous propagation in and adaptation to PBMC. To exclude this factor as a reason for the absence of macrophage tropism, we decided to clone envelope genes directly from tissues of an infected cat by PCR. We constructed 15 infectious molecular clones with envelope genes derived directly from cat tissues (bone marrow, brain, lymph node) and PBMC. All viruses containing the envelope gene sequences from PBMC, lymph node and bone marrow replicated in macrophages and PBMC. However, progeny of two (of five) clones derived from the brain showed a different phenotype: they replicated in PBMC only.

The amino acid sequences of the V3–V4 region differed between all clones analysed. These clones are likely to differ at positions in the remainder of the envelope as well, which does not allow a direct comparison as could be done between the envelope chimeras of clones 27 and 8 (Fig. 1). By comparing all V3–V4 sequences, including clones 27, 8 and 46 from the first set of experiments, we could not identify amino acid positions or potential glycosylation sites in the V3–V4 region that were specific for the exclusively lymphotropic phenotype. This suggests that, rather than an individual amino acid, the conformation of the whole envelope protein and in particular of the V3–V4 region is responsible, as evidenced by the chimeric clones 8 and 27.

Although unlikely, we cannot exclude PCR errors as the explanation for the absence of macrophage tropism. The phenotypes of these two clones are probably derived from variants with a tropism for cells of the central nervous system. None of the 15 clones replicated in primary feline astrocyte cultures or CRFK cells. For primary HIV-1 isolates and laboratory strains, tropism for macrophages and microglial cells is highly overlapping (Watkins et al., 1990), but differences in tropism for microglial cells and macrophages have also been reported for some primary isolates (Strizki et al., 1996). Our non-macrophage-tropic clones obtained from the brain may therefore represent variants that preferentially infect microglial cells and not macrophages. Interestingly, the envelope gene of the exclusively lymphotropic clone 46 was derived from the CSF isolate of FIV-UT48.

Our observations indicate that, within the FIV quasispecies, variants occur that are exclusively lymphotropic, as also shown in Fig. 4. The V3–V4 region of the envelope glycoprotein contains determinants that influence this difference in cellular tropism. Most variants, however, are dual-tropic and infect both lymphocytes and macrophages.

The FIV-14 molecular clone of the Petaluma strain was obtained from R. A. Olmsted through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAH NIH (pFIV-14 Petaluma). CRFK-HO6 cells were kindly provided by O. Jarrett, Glasgow, UK. This work was supported by the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany.

References


Macrophage tropism of FIV


