Feline immunodeficiency virus

Studies on pathogenesis and vaccine development

Feline immunodeficiency virus

Bestudering van pathogenese en vaccin ontwikkeling

Proefschrift

Ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

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Cornelis Herman Johannes Siebelink

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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>AC stage</td>
<td>asymptomatic stage</td>
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<td>ADE</td>
<td>antibody dependent enhancement</td>
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<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<td>ARC</td>
<td>AIDS related complex</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
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<td>C region</td>
<td>constant region</td>
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<td>CAEV</td>
<td>caprine arthritis-encephalitis virus</td>
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<td>CA protein</td>
<td>capsid protein</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CM</td>
<td>culture medium</td>
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<tr>
<td>Con A</td>
<td>concanavaline A</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>CrFK cells</td>
<td>Crandell feline kidney cells</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocytes</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EIAV</td>
<td>equine infectious anaemia virus</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FCaV</td>
<td>feline calici virus</td>
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<td>feline corona virus</td>
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<td>FeSFV</td>
<td>feline syncytium-forming virus</td>
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<td>fetal calf serum</td>
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<td>human immunodeficiency virus</td>
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<td>HV region</td>
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<td>HRP</td>
<td>horse radish peroxidase</td>
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<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
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<td>i.m.</td>
<td>intramuscularly</td>
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<td>integrase protein</td>
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<td>i.p.</td>
<td>intraperitoneally</td>
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<tr>
<td>Ig</td>
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<td>LPS</td>
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<td>LTR</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>NC protein</td>
<td>nucleocapsid protein</td>
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<tr>
<td>NK cells</td>
<td>natural killer cells</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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PCR                     polymerase chain reaction
PGL                     persistent generalized lymphadenopathy
PLV                     puma lentivirus
PND                     principal neutralizing determinant
PR protein              protease protein
PWM                     pokeweed mitogen
rhIL-2                  recombinant human interleukin-2
RNA                     ribonucleic acid
RRE                     rev responsive element
RT protein              reverse transcriptase protein
rVV                     recombinant vaccinia virus
SIV                     simian immunodeficiency virus
SPF                     specified pathogen free
SU protein              surface protein
TM protein              transmembrane protein
V region                variable region
VN                      virus neutralization
Chapter 1

General Introduction

Partially based on: Genome organization and genetic variation of feline immunodeficiency virus

Marnix L. Bosch, Kees H.J. Siebelink, and Albert D.M.E. Osterhaus
Feline immunodeficiency virus (FIV) is classified as a member of the genus Lentivirus (subfamily Lentivirinae) of the Retroviridae family on basis of its morphology, biochemical characteristics, genomic organization, Mg$^{2+}$ dependent reverse transcriptase, and nucleotide sequence homology with other members of this genus. Lentiviruses cause chronic, lifelong infections in their respective host species, which may be followed by a slowly progressive and degenerative disease (2,16,53,104,108,115,199,236,248,289). FIV was first isolated from a domestic cat suffering from an immunodeficiency syndrome reminiscent of AIDS in humans (199). Subsequently it was shown that upon FIV infection, domestic cats may develop an immunodeficiency syndrome, hallmarked by secondary and opportunistic infections. The clinical stages preceding the final stage of feline AIDS, are similar to those observed in humans with HIV infection: the acute stage is followed by the asymptomatic carrier (AC) stage, after which a persistent generalized lymphadenopathy (PGL) gradually leads to the stage of AIDS related complex (ARC) (116). Then the animal develops full blown AIDS. The different stages of FIV infection of cats may often not be quite distinct and rapid transitions between the respective stages may be observed (see below).

FIV infections occur virtually worldwide in domestic cats and to date three viral clades (A, B, and C) with partially overlapping geographical distributions have been identified on basis of env sequences (245). This indicates that FIV infection of domestic cats has occurred for a long period of time. Recent serological and molecular phylogenetic studies have shown the occurrence of infections with FIV or closely related lentiviruses in zoo collections and in free ranging populations of non-domestic felid species (190). Molecular phylogenetic analyses of the gag and pol genes showed that FIV is more related to the ungulate lentiviruses (Fig. 1). However, the pathogenesis of FIV infection shows more resemblance to that of primate lentivirus infections.

**The virus**

*Mature lentivirus particles are spherical to ellipsoid particles of approximately 100 - 125 nm in diameter with a bar- or cone shaped core (Fig. 2; 199,288). The core is composed of viral capsid (CA) proteins of 24 kD (p24). Inside this core two identical positive RNA strands are found, with which the enzyme reverse transcriptase (RT) and nucleocapsid (NC) protein of 7 kD (p7) is closely associated.*
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The viral surface, the envelope, is a lipid bilayer membrane which is derived from the infected cell through a budding process. The inner side of the membrane is covered by the matrix protein (MA) that helps providing the shape of the virus particle (49). The CA-, the MA-, and the NC proteins are derived from one Gag precursor protein of 55 kD (57,62,253). The envelope glycoproteins cover the viral membrane. They consist of a transmembrane (TM) protein of 41 kD (gp41) and a surface (SU) protein of 95 kD (gp95) in the case of FIV. They are derived from a envelope precursor protein of 160 kD, which is first processed by trimming and cleavage of the leader protein resulting in a 135 kD protein (254,276). The TM glycoprotein is anchored in the membrane. This results in the presentation of the central and N-terminal part outside the virion. The SU glycoprotein is attached to the TM glycoprotein by non-covalent bounds (100).

Figure 1. Phylogenetic tree based on nucleotide sequences of lentiviruses pol and gag genes. Data were extracted from GenBank databases: FIV Petaluma (M25381), FIV PPR (M36968), FIV TM2 (M59418), FIV Puma (U03982), FIV Utrecht 113 (x68019), HIV-1 BH5 (K02012) HIV-2 SBLISY (G04498), SIVmac32H (D01065), Visna Iceland 1514 (M10608), CAEV Clement (M33677), EIAV clone 1369/409-2 (M16575), and BIV HXB3 (U32690). Sequences coding for the Pol- and Gag polyproteins were aligned using the PileUp program of the GCG software package (79) (version 8.0) with a gap creation penalty value of 3.0 and gap extension limits of 0.1, respectively. Distances were calculated according the Kimura-2-parameter equation and the tree constructed utilizing the neighbour-joining method (124). Branches are drawn proportionally to the relative genetic distances as indicated by the bar. For phylogenetic analysis the software package MEGA release 1.01 (134) was employed.
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Genome organization

The proviral genome of FIV is approximately 9500 bp in length and is bordered by two long terminal repeats (LTR). (Fig. 3) Three large open reading frames (ORF), gag, pol and env encode structural and enzymatic proteins. Several short ORFs (sORFs) or short exons of multiple spliced mRNAs encode for virus regulatory and accessory proteins which have a direct or indirect influence on virus replication (for a review, see reference 61,174).

LTR. The LTRs direct and control viral DNA and RNA synthesis and the integration of proviral DNA into the genome of the host cell. The length of the LTRs of FIV, approx. 350 bp, is comparable with those of CAEV, Visna virus and BIAV, and is significantly shorter than the LTRs of the primate lentiviruses (101,173,189,237,238,252,259). Within the FIV LTR a number of regulatory sequences like TATA box, polyadenylation signal and polyadenylation site and enhancer sequences can be found, although not all of these features are conserved (249).

Figure 2. Electron micrograph of FIV budding from infected cells (A, magnification 38,000), virus particles (B, magnification 62,000), and schematic representation of FIV (C).
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Gag. The gag gene is approximately 1350 bp in length and encodes a Gag precursor protein of 55 kD (p55), which is cleaved into the MA- (p15), CA- (p24) and NC- (p7) proteins (57,62,253). Alignments of the FIV gag gene sequences with those of the gag genes of other lentiviruses have revealed significant homologies in the respective Gag proteins, ranging from about 49% amino acid identity with the EIAV gag gene products, to about 40% amino acid identity with those of the HIV-I Gag protein (57,61,189). Antibodies directed against the Gag proteins of FIV and EIAV proved to be reciprocally cross reactive in ELISA and Western blot assays (57). The gag gene is rather well conserved within FIV strains: The highest degree of conservation is observed for the CA protein and most of the variation is observed for the NC protein (157).

Gag-Pol frameshift. The gag and pol gene reading frames of FIV partially overlap (Fig. 3) with the pol being in the -1 reading frame of the gag gene (178). Two consensus sequences for frameshifting have been observed in the overlapping region (GGGAAAC and GAGAAAC resp.) and similar sites are also found in the homologous regions of type D retroviruses and Visna virus (205,247). Mutational analyses have demonstrated that frameshifting occurs in the GGGAAAC sequence, possibly at the asparagine codon (AAC) within this sequence (178). In this study the need for RNA pseudoknot formation downstream of this signal sequence was also demonstrated.

Pol. The pol gene is approximately 3360 bp and encodes a precursor polyprotein. Comparative analysis of the pol sequence with known amino-termini of HIV-I pol products (146) identifies the predicted cleavage sites of the FIV Pol precursor (62). The resulting cleavage products are the (predicted) protease (PR: 123 amino acids), RT (559 amino acids), endonuclease or integrase (IN: 278 amino acids) and a protease-like protein of 131 to 132 amino acids is located between RT and IN (165). Computer searches have identified this latter protein as a putative dUTPase (167). The dUTPase of FIV is required for efficient growth in primary PBMC (279). Such a dUTPase-like sequence is present in EIAV and Visna virus but not in the primate lentiviruses HIV-1, HIV-2 and SIV (28,60).

Env. In the primate lentiviruses the env gene products control many functions which are important for viral pathogenesis and also for vaccine development (for a review, see reference 143). They contain the binding site for the cellular receptor CD4 (142,191), the fusion domain involved in entry of the virus into target cells and in syncytium formation (27,74,88,133), and as yet unidentified regions controlling the viral host range and cytopathicity (10,38). Furthermore they contain neutralizing determinants, most notably in the V3 region (76,118) through which antibodies can interfere with viral infection of target cells.
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The FIV env gene codes for a precursor protein of approx. 843 amino acids containing multiple potential glycosylation sites (254). A highly hydrophobic putative leader sequence is found at amino acid position 156 through 186 which would put the start of the SU protein around position 190 and would identify the preceding region as a potential homologue of the L gene of Visna virus (49,130,254,276). Around amino acid position 618 (depending on the isolate) a consensus sequence for susceptibility of a trypsin-like enzyme is found, similar in position and sequence to those found for the primate lentiviruses, in which cleavage of the Env precursor is indeed demonstrated at this position (275). The cleavage products most likely correspond to the gp95 and gp40 glycoproteins observed on protein gels of FIV or FIV-infected cells (253,254). The env gene is clearly more variable than the gag and pol genes (86). It has been noted previously that variation in the env is not randomly distributed (173,179,198,204) and this has led to the definition of five hypervariable regions: HV1 to HV-5. Like HIV-1, FIV can be divided in different subtypes. Based on the genetic diversity pattern of the region encompassing the variable regions HV3, HV4, and HV5, three distinct envelope sequence subtypes are presently recognized (245).

- Other ORFs. In the original papers on the molecular cloning of FIV it was noted that the sequence of FIV, like those of the primate lentiviruses, contains a number of open reading frames (ORFs) which might be attributed to regulatory genes (179,188,189,204,259). Extensive homology searches have not revealed similarities between these ORFs and the regulatory gene sequences of HIV and SIV. Furthermore not all of the initially noted ORFs could be found in full length FIV clones from later dates. Four of the additional ORFs are presented here: the presumed vif, rev, A ORF and H ORF (also designated B ORF).

Vif. The location, size and function of the presumed vif gene is similar to that of the primate lentiviruses (266) The vif gene encodes a hydrophobic and basic protein and it is highly conserved in different FIV isolates (157,204,259). Its degree of conservation between the different FIV isolates is similar to that observed for gag. Vif deletion mutants of FIV produced virion associated reverse transcriptase activity in CrFK cells upon transfection, but cell free virus did not infect feline CD4+ cells indicating that, as was shown for various primate lentiviruses, vif activity of FIV is essential for cell free infection (266).

Rev. The Rev protein is encoded by a multiple spliced mRNA of which the first coding exon is located in the L domain of the env ORF and the second exon is probably identical to the H ORF at the 3' end of env extending into the 3' LTR (125,203,267). Rev is a regulatory protein that is translocated into the nuclei of infected cells where it binds to specific cis-acting target sequences in the viral
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mRNA before splicing, termed the Rev responsive elements (RRE), and it facilitates the translocation of full length or single spliced viral mRNAs from the nucleus to the cytoplasm (159). The RRE of FIV is mapped to a 243 bp region at the 3' end of env (160,203). In other lentiviruses the RRE is found in the env gene at the SU-TM junction (37,158,224). At low concentrations of Rev in the cytoplasm of infected cells the viral mRNAs are predominantly small and multiple spliced (63,68). Multiple spliced mRNAs encode regulatory proteins. At high concentrations of Rev in the cytoplasm, the viral mRNAs are predominantly full length or single spliced. Full length or single spliced viral mRNAs encode structural proteins resulting in the production of virus particles. Thus the concentration of Rev plays a pivotal role in determining whether a lentivirus infection is latent or productive.

A ORF. The A ORF is located between the pol- and env gene in a region, where in the primate lentiviruses the first exon of tat (transactivator) and rev are found. It is conserved in most FIV clones, although it is interrupted in the 34TF10 clone (204). The 34TF10 clone is replication competent which indicates that the A ORF product is dispensible for virus replication. Using A ORF frameshift mutants of FIV it was shown that replication and propagation of these mutant viruses was reduced in established T cell lines and that the productive infection of primary
PBMC with these mutant viruses was severely reduced when compared to wild type virus (265). This suggests that the ORF \( A \) gene enhances viral replication in primary PBMC.

**H ORF.** The \( H \) (or \( B \)) ORF is found in all FIV clones directly 3’ of env, where the \( nef \) gene is located in the primate lentiviruses (204). This ORF is longer in the molecular clone pFTM19 due to an extension at the 5’ end. In all clones the \( H \) ORF contains a stretch of basic amino acids reminiscent of a nuclear or nucleolar localization signal which is also found in HIV-1 and HIV-2 Rev (52,103). This finding is therefore compatible with the involvement of the \( H \) ORF in FIV rev gene activity (see above).

**Pathogenesis and epidemiology**

Like in the clinical course of HIV infection in humans, five different stages can be recognized in the clinical course of FIV infection in cats (116). Specified pathogen free (SPF) cats experimentally infected with FIV develop a transient low grade fever, neutropenia and generalized lymphadenopathy (201,289). The fever and neutropenia may persist for some days up to several weeks while the generalized lymphadenopathy can persist for several months. Most cats recover from these acute symptoms (16,18,33,80,269,285). During this period the cats seroconvert and serum antibodies to the structural proteins can be demonstrated, which may be used for diagnostic purposes (50,109,150,277). The acute phase is followed by the AC stage in which no apparent clinical symptoms are observed. This phase can last for years. In HIV infected humans this stage is followed by a phase characterized by PGL. Only few infected cats have shown to develop PGL (116), which indicates that in FIV infection this phase may exist, but that the duration is relatively short and that the illness proceeds fast to the next stage of ARC in those cats that develop disease. This is characterized by weight loss, chronic diarrhea, stomatitis, gingivitis, upper respiratory tract infections and skin infections. Within several months to a year this stage leads to AIDS. Cats with AIDS suffer from severe immunodeficiency symptoms like those of the previous stage aggravated by opportunistic infections, emaciation, anemia, lymphopenia, neutropenia, thrombocytopenia, tumours, and neurological disorders (116).

Before the onset of clinical signs, during the acute stage of infection, high numbers of infected cells are present in lymphoid organs, including the thymus, bone marrow, lymph nodes, spleen, tonsil, and lymphoid tissues of the intestinal tract (18). Like HIV and SIV, FIV appears to selectively infect T cells and cells of
the monocyte/macrophage lineage (19,29,30,65,201). It has been shown that FIV can infect other cells than CD3+ cells, like B cells, astrocytes and megakaryocytes in vivo and feline kidney cells (CrFK cell line) in vitro (41,53,65,289). However, the cat CD4 molecule does not seem to function as the primary receptor for FIV (111). Another cell surface molecule, the feline homologue of CD9, has recently been identified as the most likely candidate for an FIV receptor (283). Human CD9 has been implicated as a signal transduction molecule and is expressed on the surface of a wide range of haematopoietic cells. This may explain why FIV can infect a wide variety of cell types, both in vivo and in vitro.

In line with HIV infection in humans a selective loss of CD4+ lymphocytes is observed in cats infected with FIV (2,16,80,104,184,269). Within six months after infection a decrease in percentages and numbers of circulating CD4+ lymphocytes and in the CD4+/CD8+ T cell ratio is observed in SPF cats, experimentally infected with the Petaluma strain of FIV (269). CD4+/CD8+ ratio inversions have been reported to occur early (<3 months) (268) or later in the infection (18 - 24 months) (2,16). A rapid expansion of the CD8+ lymphocyte subpopulation, which can occur within 4 weeks post infection, contributes to the CD4+/CD8+ inversion (282). The depletion of CD4+ lymphocytes may be the result of a direct clearance of the infected CD4+ cells or of uninfected CD4+ cells loaded with circulating gp120, by cytotoxic T cells (CTL). Also the direct cytotoxic effect of FIV as a result of intense virus budding causing changes in membrane integrity, syncytium formation, apoptosis (22,186), and accumulation of unintegrated viral DNA may cause depletion of CD4+ cells. Early precursors of CD4+ cells could also be infected, which may lead to a reduced quantity of fresh mature lymphocytes (71).

Epidemiological studies have revealed a worldwide distribution of FIV. Serological surveys of domestic cats with symptoms reminiscent of an immunodeficient syndrome, showed a varying incidence from 3% - 14% in Europe and the United States through 44% in Japan (15,117,152,201,248,285). The prevalence of FIV infection in the overall domestic cat population worldwide ranges between 1% and 30%. The large variations of prevalence in the different countries may reflect differences in life styles of cats. In general the older free roaming male cats have the highest incidence of FIV infection. Cross reactive antibodies to FIV were common in several free ranging populations of large cats, including the East african lion (Panthera leo), the cheetah (Acinonyx jubatus), the puma (Felis concolor) and the bobcat (Lynx rufus) and in several zoo populations including lions, cheetahs and pumas (190). No apparent clinical symptoms are observed in these large cats. Molecular phylogenetic comparison of the pol genes of the puma lentivirus (PLV) and FIV, and the antigenic relationship showed that the PLV
isolates are more related to FIV than to other lentiviruses (140,190).

FIV can be isolated from blood, plasma, cerebrospinal fluid and saliva of infected cats (53,163,289). Although the modes of transmission are not fully understood, the presence of infectious FIV in the saliva suggests bite wounds may account for a major proportion in the transmission (248,285). This can explain the higher incidence in free roaming male cats, which are known to express more territorial aggression. The vertical transmission of lentiviruses may occur in utero during the intrapartum period, or in the early post natal period (3,40,59,145,164, 226,260). Although in utero infection can not fully be excluded, it seems to be uncommon in FIV infection (32,273). FIV can be transmitted via milk during acute maternal infection and much less efficiently or not during chronic maternal infection (233).

**Humoral immune response**

Within eight weeks after infection almost all FIV infected cats seroconvert (13,58,72,109,185). Serum antibodies directed to the Env glycoprotein can be demonstrated first, followed by antibodies to the Gag protein. Antibodies to non structural proteins can also be demonstrated. At present no data are available on the presence and kinetics of different (sub)classes of immunoglobulins directed to FIV proteins upon FIV infection.

Since the Gag proteins are well conserved among the different FIV strains, antibodies to the Gag proteins are valuable for routine diagnostic screening of FIV infection in cats using ELISA systems. Like in HIV infection in humans, confirmation of the ELISA data can be performed by more expensive and time consuming assays like western blot analysis, PCR and virus isolation. Like in humans and monkeys exposed to HIV and SIV respectively, it was shown that in persistently seronegative cats, which were housed together with FIV infected cats, FIV DNA could be detected in their PBMC and bone marrow cells by PCR (46).

Within the envelope glycoprotein, several antigenic sites reactive with sera from FIV infected cats can be found (13,50,56,72,148,197). Three linear immunodominant epitopes have been mapped: The first within the HV3 region of SU, the second at the COOH terminus of SU and the third on the TM2 region of the transmembrane protein. These immunodominant sites are reminiscent of HIV-1 in which at similar positions immunodominant sites have been described (for a review, see reference 143). There are indications that antibodies directed to the envelope protein not only neutralize but may also enhance FIV infection in vivo and
in vitro (110,221-223).

**Virus neutralizing antibodies**

Virus neutralizing serum antibodies arise soon after infection and are generally believed to play a role in the protective immune response against lentiviruses. The major target of lentiviruses to which neutralizing antibodies are directed is the SU protein. The SU protein of HIV contains several regions that are involved in neutralization, including the principle neutralization determinant (PND) in the V3 loop, the CD4 binding site and sites in the second conserved (C2) and V2 regions (10,103,208,264, for a review, see reference 143). Mutations within or outside these regions may render the virus resistant to virus neutralizing antibodies (169,214,284, for a review, see reference 187). Continuous variation of the envelope glycoprotein sequences and of the subsequent antibody response are observed (4,11,31,119,176, 182). On the SU protein of FIV a linear immunodominant neutralizing determinant has also been mapped to the HV3 region (50,56,148).

Detection of virus neutralizing antibodies in vitro is largely dependent on the virus and cell substrate used (14). Using CrFK cells and CrFK adapted virus strains high titers of broadly neutralizing antibodies have been detected in the sera of naturally and experimentally infected cats. In contrast, in VN assays using primary virus isolates and Con A stimulated T cells or T lymphoid cell lines, no or only low titers of highly type specific neutralizing serum antibodies were demonstrated (14,243). Similar results have been obtained with HIV virus neutralization assays when different cell substrates and cell line adapted vs non adapted viruses were used (230). Since T cells are natural host cells for FIV (18), their use in VN assays seems more likely to reflect the in vivo situation than the use of CrFK cells and CrFK adapted viruses in these assays. The mechanisms underlying these differences are still unclear. Immunization of cats with the immunodominant epitope of the V3 region of the SU protein was shown to elicit serum antibodies that proved to be broadly neutralizing in the CrFK assay. However, these antibodies did not confer protection against FIV infection (149).

**Virus enhancing antibodies**

Antibody dependent enhancement (ADE) of viral infection is a phenomenon by which antibodies directed against the virus facilitate and enhance the process of infection (for a review, see reference 161,173). It was described that immunization with inactivated whole FIV may elicit enhancing antibodies (110). At least two different mechanisms of ADE have been described for lentiviruses (107,121,175, 221-223,258). One described mechanism operates through Fc receptors on
macrophages which act as a receptor for immunoglobulin complexed with virus. Once internalized via this mechanism, the virions are not effectively inactivated and can replicate within the macrophage. Another mechanism of ADE operates via the activation of complement by the virus-antibody complex: complement-mediated ADE (C'-ADE). The complement-antibody-virus complex binds to complement receptors on cells, and the complex is subsequently internalized. Both types of ADE has been reported for a number of viruses (for a review, see reference 161,257). Recently a third mechanism of ADE has been described for HIV-1, in which the same antibodies either neutralized or enhanced HIV-1, dependent on the phenotype of the virus involved. Polyclonal and monoclonal antibodies directed to different domains of the envelope glycoprotein may neutralize one HIV-1 strain but enhance another (94,128,232). Titers of enhancing antibodies in sera from HIV-1 infected humans may be extremely high (up to 1:65,000), which may cause a dramatic reduction or abolishing of the neutralizing activity measured against HIV-1.

Cellular immune response

Despite the induction and persistence of virus neutralizing serum antibodies (271, 272), lentiviruses cause a persistent infection in their respective hosts. Cell-mediated immune mechanisms, especially natural killer (NK) cells and cytotoxic T cells (CTL) but also the T-helper cell responses have been shown to be important in the clearance of virus and in the control of persistent virus infections. NK cells recognize and kill infected cells in a non MHC restricted manner and may play an important role in the clearance of HIV infected cells in humans. Before the onset of clinical symptoms mitogen- and antigen induced proliferative T cell responses are decreased (2,16,94,269). During the acute FIV infection the primary and secondary T cell responses are impaired (23,24). To date no data of NK activity or FIV specific T helper cell responses are available in FIV infected cats.

FIV specific CTL have been detected in peripheral blood of experimentally infected cats, 7 to 9 weeks post infection. These cells proved to be predominantly CD8+, which indicates that the lysis of FIV infected cells is, at least in part, MHC class I restricted (246). CD8+ CTL’s have been detected in the peripheral blood of 3 out of 4 cats immunized with 17-mer peptides (70). These CTL’s lysed autologous and not allogeneic skin fibroblasts pulsed with a homologous 10-mer peptide. An antibody response and proliferative response to the 17-mer peptide were also measured in vitro in these cats, which indicates a class II MHC restricted T cell response. For HIV it has been shown that CD8+ cells can suppress the replication of
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HIV in CD4\(^+\) cells \textit{in vitro} without killing these cells. This antiviral activity of CD8\(^+\) cells has not been identified in FIV infected cats.

Vaccine development

Although it is not known at present what correlates with protective immunity in lentivirus infections, the ideal vaccine probably should induce both a humoral and cellular immune response. The induced antibodies should preferably neutralize a wide variety of virus strains and therefore be preferably directed to conserved neutralizing sites. Furthermore the induction of infectivity enhancing antibodies should be avoided (for a review, see reference 161,257). Since the most common natural mode of infection in cats is probably not via mucosal surfaces (285), the induction of mucosal immunity, which is supposed to be of major importance in the prevention of HIV-1 infection, may be less relevant in the prevention of FIV infection of the cat. The induction of a potent T helper cell response may be a prerequisite for the generation of protective and long lasting antibody and CTL responses. To date, virtually all the classical and modern approaches for the development of viral vaccines have been or are being explored for the development of lentivirus vaccines, with varying degrees of success (for a review, see reference 35,217). Probably, the most successful vaccination studies in a lentivirus system have been carried out in the FIV-cat system: cats vaccinated with paraformaldehyde fixed T cells (FL-4 cells) persistently infected with FIV, and cats vaccinated with paraformaldehyde inactivated FIV derived from the same cells, proved to be protected from homologous and to a lesser extent from heterologous FIV challenge infection (286,287). The protective immunity could be transferred to naive cats with the plasma of thus vaccinated animals, indicating that FIV specific antibodies were at the basis of the induced protection (105). Although these experiments could be confirmed by others with the same vaccine preparations, a similar approach with vaccine candidates produced in other cells was unsuccessful in several experiments (110,278). In one of these experiments, the induced response even seemed to predispose for enhancement of infection rather than for protection. Experiments with non-replicating subunit vaccines, predominantly containing the envelope glycoproteins of FIV or selected domains, all failed to show the induction of protective immunity (110,149). Similar approaches have yielded a certain degree of success in the SIV/macaque- and HIV-1/chimpanzee system (20,34,75,82,120,192,193,211,251). When this approach was combined with the use of live recombinant viruses expressing the envelope glycoproteins, the induction of protective immunity
proved to be more efficient in the SIV-macaque systems (113). So far this approach has not been studied in the FIV-cat system. The use of attenuated lentiviruses (47,209), like the nef gene deleted or mutated viruses, which proved to be successful in the SIV-macaque system, has also not been documented in the FIV cat system.
Outline of this thesis

After the description of the present knowledge of FIV and FIV infection of cats in this chapter, chapter 2 describes the establishment of the FIV model system used in this thesis for studying aspects of the pathogenesis of FIV infection and for the evaluation of different vaccination strategies. In this chapter it is shown that infected cats develop a mild immune suppression, serological assays are described, and a series of molecular clones are generated directly from the bone marrow DNA of a naturally infected cat in The Netherlands. These clones are used in chapter 3 to identify determinants involved in the tropism of FIV to replicate in CrFK cells, which are continuously growing non-lymphoid cells, originating from feline kidneys. These molecular clones of FIV are also used to identify mechanisms of escape from VN activity of serum antibodies from experimentally infected cats at the molecular level. In chapter 4, the generation and evaluation of a candidate subunit vaccine for the induction of FIV specific immunity is described. To this end FIV envelope glycoproteins are produced in a recombinant vaccinia expression system and subsequently formulated in an iscom presentation form. After the demonstration of the induction of FIV specific antibodies in cats with this and other FIV envelope protein preparations, the results of a parenteral challenge with homologous FIV strain in thus vaccinated cats are described. In the last chapter, the results of the experiments carried out in the framework of this thesis are summarized and discussed.
Chapter 2

Establishment of the FIV Infection Model
Feline Immunodeficiency Virus (FIV) Infection in the Cat as a Model for HIV Infection in Man:
FIV-Induced Impairment of Immune Function

Kees H.J. Siebelink, I-Hai Chu, Guus F. Rimmelzwaan, Kees Weijer, Rob van Herwijnen, Peter Knell, Herman F. Egberink, Marnix L. Bosch and Albert D.M.E. Osterhaus

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Abstract

To assess the value of feline immunodeficiency virus (FIV) infection as a model for human immunodeficiency virus (HIV) infection in man, we studied the impairment of certain immunological functions following natural or experimental FIV infection. Proliferative responses of peripheral blood mononuclear cells (PBMC) from symptomatic and asymptomatic cats after naturally or experimentally acquired FIV infection, induced by activation with the mitogens concanavalin A, pokeweed mitogen or lipopolysaccharide or by stimulation with human interleukin-2 (IL-2), were significantly lower than the proliferative responses found with PBMC from non infected control cats. Also IL-2 production levels of mitogen-activated PBMC from naturally infected symptomatic cats were significantly reduced. These data confirm that the pathogenesis of FIV infection in the cat, like HIV infection in man, is characterized by a serious malfunction of the immune system.

Introduction

Since the discovery of human immunodeficiency virus type 1 (HIV-1) as the cause of acquired immunodeficiency syndrome (AIDS) in 1983 (17,77), it was realized that there is an urgent need for animal models to study the pathogenesis of HIV-1 infection and possibilities for interventional strategies. Apart from chimpanzees and gibbons no animal species can be infected with HIV-1, and these apes do not develop clinical symptoms similar to AIDS upon experimental infection (9,151). However, it has been shown that certain monkey species can be infected with HIV-2 and with a number of simian immunodeficiency viruses (SIV), and that they may subsequently develop an AIDS-like syndrome (for a review, see reference 250). Recently, a lentivirus of the cat, feline immunodeficiency virus (FIV), has been identified in cats with an AIDS-like syndrome (199). Since cats are less expensive and easier to handle than monkeys, the FIV infection model may be of special interest for testing the potential of antiviral drugs and vaccination strategies in relation to HIV infection in man.

The knowledge about the pathogenesis of FIV infections is limited at present (for review see references 200,248). Although infection under natural circumstances seems to be associated with the development of chronic disease symptoms...
Establishment of FIV infection model

eventually leading to AIDS-like disease, it is difficult to reproduce the disease by experimental infection of cats (289). Therefore, we have now undertaken studies regarding the functioning of the immune system of cats following natural or experimental infection with FIV. The progression to AIDS in man following HIV-1 infection is characterized by the gradual impairment of in vivo and in vitro immune functions (112,225,262). Specifically, loss of mitogen-induced lymphocyte proliferation is seen shortly after infection in asymptomatic individuals (172,263). In this report, we present studies on mitogen and interleukin-2 (IL-2) induced proliferative responses and IL-2 production of peripheral blood mononuclear cells (PBMC) of cats after infection with FIV.

Materials and Methods

FIV-infected and control animals

Four different groups of cats were used in these studies. Fifteen private household cats were identified as seropositive for FIV in an enzyme-linked immunosorbent assay (ELISA) (239). Five of these cats (3-9 years old), designated Group S, showed clinical signs of chronic disease suggestive for feline AIDS (289). The other ten (2-6 years old), designated Group A, were healthy at the time of sampling. Twelve specific pathogen-free (SPF) cats (1.5-3 years old), designated Group E, had previously been experimentally infected subcutaneously with a Dutch field isolate of FIV (n=10) or the Petaluma strain (n=2) (199), 12 to 30 months before sampling. These animals were negative for antibodies against a number of viruses which commonly infect cats, including feline leukemia virus (FeLV), feline panleukopenia virus (FPV), feline calici virus (FCaV), feline herpes virus-1 (FHV-1), feline syncytium-forming virus (FeSFV) and feline corona virus (FCV) as demonstrated in regular serological screening procedures. The last group consisting of 25 private household cats (1.5-10 years old) seronegative for FIV, was designated Group C and served as a noninfected control group. Comparison of the data obtained in Group C for proliferative responses and IL-2 production upon mitogen stimulation to data obtained previously in our lab for a group of SPF cats (n=4) demonstrated no significant differences between these two groups (data not shown). Therefore, only non-SPF cats were used as controls in these assay systems.

Sampling

Blood samples were collected once from cats of Group S and Group A. From 12 experimentally infected cats (Group E) blood samples were collected monthly for
determination of lymphocyte counts and once, 0.5 to 2 years after infection, to assess the mitogen and IL-2-induced proliferation and IL-2 production of PBMC. Peripheral blood mononuclear cells (PBMC) were isolated from preservative-free heparinized blood by density gradient centrifugation on Ficoll-Isopaque. The cells were washed three times with RPMI 1640 (Gibco, Grand Island, NY), supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamin (2mM) and β-mercaptoethanol (2.10⁻⁵ M), hereafter designated as culture medium (CM). The lymphocyte counts from the experimentally infected cats were determined monthly. The lymphocyte counts from the three other groups of cats were determined once.

**Mitogen-induced proliferation**

PBMC (10⁵ cells/well) were cultured at 37°C, 5% CO₂, in a humid environment in CM, supplemented with 10% heat-inactivated calf bovine serum (FCS) with concanavalin A (ConA (5 µg/ml) (Flow Laboratories) or pokeweed mitogen (PWM) (1 µg/ml) (Flow Laboratories) for 72 h or lipopolysaccharide (LPS) (50 µg/ml) (Escherichia coli 0.27:B8, Difco Laboratories) for 96 h in round-bottomed tissue culture plates using unstimulated cells as a control. The optimum concentration of mitogens and time of culture were first defined by dose-response curve. Proliferative responses were measured by [³H]-thymidine incorporation (1 μCi/culture). Results presented are the mean of triplicate wells. Differences were found significant for \( p < 0.01 \) as calculated according to the Student’s \( t \)-test.

**Proliferative response to exogeneous IL-2**

The response of PBMC to exogeneous IL-2 was tested using a method similar to the one described by Goitsuka et al. (83) PBMC (10⁶ cells/ml) were incubated with ConA (2 µg/ml) for 1 h at 37°C in CM, supplemented with 10% FCS in 12 x 75 mm culture tubes (Falcon, catnr. 2085, Becton and Dickinson, E. Rutherford, NJ). The cells were washed three times with CM and cultured for 96 h with 100 IU recombinant human IL-2/ml (rIL-2) (Boehringer Mannheim, Germany) in 96 wells flat-bottomed tissue culture plates. Sixteen hours before harvesting, the cells were pulsed with [³H]-thymidine to measure their rate of proliferation, using unstimulated cells as control. The stimulation index was calculated by dividing the cpm of the IL-2 stimulated culture by the cpm of the unstimulated culture. Differences were found significant for \( p < 0.01 \) as calculated according to Student’s \( t \)-test.

**IL-2 production**

Production of IL-2 was measured using the method described by R. Goitsuka et
Establishment of FIV infection model

PBMC (10^5/well) were cultured with or without ConA (10 μg/ml) in CM supplemented with 10% FCS in 96-well round-bottomed culture plates. After 24 h, the culture supernatants were tested for the presence of IL-2, using an IL-2-dependent cloned murine cytotoxic T cell line (CTLL): CTLL cells (5.10^5), in the logarithmic phase, were seeded into 96 wells of round-bottomed microtiter plates. Twofold serial dilutions of the culture supernatants were added. After three days the cells were pulsed with [3H]-thymidine and after 16 h harvested and counted in a scintillation counter (LKB). The units of IL-2 were determined by probit analysis at 50% of the rIL-2-standard (Boehringer). In preliminary experiments optimal conditions for cell concentration, dosage of mitogens, and culture time were determined. Differences were found significant for p <0.01 as calculated according to Student's t-test.

Results

Mitogen-induced proliferative responses of PBMC

To assess the effect of FIV infection on the immune system, we studied the proliferative responses to various mitogens of PBMC from symptomatic and asymptomatic cats infected with FIV. The data are presented in Figure 1. ConA-induced proliferative responses of PBMC from five naturally infected cats with clinical symptoms (Group S) were significantly lower than the responses of PBMC from 25 control cats (Group C). Also for 10 naturally and 12 experimentally infected FIV seropositive cats without apparent clinical symptoms (Groups A and E), significantly lower proliferative responses were found. The geometric means of the values found for the control group, the symptomatic and the two asymptomatic seropositive groups were 75,769, 5,151, 35,834 and 33,908 cpm, respectively (Fig. 1A). Similar results were found for PWM- and LPS-induced proliferative responses: PBMC from the 5 naturally infected cats with clinical symptoms and from the 10 naturally and the 12 experimentally infected asymptomatic cats showed significantly lower PWM- and LPS-induced proliferative responses than the PBMC from the 25 control cats. The geometric means of the values found upon stimulation with PWM for the control group, the symptomatic, and the two asymptomatic seropositive groups were 31,918, 1,159, 14,348 and 10,829 cpm, respectively (Fig. 1B). Following stimulation with LPS the geometric means of the values found for the control group, the symptomatic, and the asymptomatic seropositive groups were 8,399, 1,286, 4,802 and 2,382 cpm, respectively (Fig. 1C).
Figure 1. Mitogen-induced proliferative responses of PBMC from the control (C), naturally infected symptomatic (S), and naturally infected asymptomatic (A) and experimentally infected asymptomatic (E) cats. Proliferative responses induced by ConA (A), PWM (B) and LPS (C) are expressed as counts per minute of tritiated thymidine incorporation.
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**IL-2 production and proliferative responses to exogenous IL-2**

The impaired responses to mitogens in FIV-infected cats could be due either to reduced IL-2 production or to a reduced response to the IL-2 produced. Therefore IL-2 production and proliferative responses to exogeneous IL-2 by PBMC from cats were measured.

ConA-induced production of IL-2 by PBMC from the 5 naturally infected cats with clinical symptoms and from the 12 experimentally infected cats was significantly lower than the production of IL-2 by PBMC from the 25 control cats. No significant differences were found when the ConA-induced production of IL-2 by PBMC from the 10 naturally infected cats without apparent clinical symptoms were compared with the IL-2 production by PBMC from the 25 control cats. The geometric means of the IL-2 production found for the control group, the symptomatic seropositive group, and the two asymptomatic groups were 13, 1, 16 and 9 IU IL-2/ml, respectively (Fig. 2). No IL-2 production (< 0.2 U/ml) could be measured in the unstimulated cultures.

![Figure 2. IL-2 production, after stimulation with ConA, of PBMC from control (C), naturally infected symptomatic (S), naturally infected asymptomatic (A) and experimentally infected (E) cats. Results are expressed as international units (IU) of IL-2 per ml.](image)

The proliferative responses of ConA-activated PBMC from the 5 naturally infected cats with clinical symptoms to a saturating amount of human recombinant IL-2 (100 IU IL-2/ml) were significantly lower than the responses of the PBMC from the 25 control cats. Also, for PBMC from the 10 naturally and the 12 experimentally infected asymptomatic cats, significantly lower proliferative responses to IL-2 were found. The geometric means of the control group increased from 5,917 cpm to 25,088 cpm upon cultivation in the presence of IL-2 (Fig. 3A). For the naturally and experimentally infected seropositive nonsymptomatic cats, these values increased from 1,153 to 12,958 cpm and from 3,466 to 18,522 cpm, respectively.
The geometric mean cpm of the PBMC from naturally infected symptomatic cats was 139 cpm and did not increase significantly when the cells were cultured in the presence of IL-2. The stimulation indices of these cultures were 4.2, 11.3, 5.4 and 1.4 respectively. Similar results were obtained when PBMC were not activated with ConA prior to the addition of IL-2, although the differences in proliferative responses found between the respective groups were less pronounced but the stimulation indices remained the same (Fig. 3B).

**Figure 3.** Proliferative responsiveness of PBMC from the four groups of cats, in cultures with and without recombinant human IL-2 (100 IU/ml). Proliferative responses after stimulation with ConA (3A) or without ConA (3B) are expressed as counts per minute of tritiated thymidine incorporation. Control Group (C); naturally infected symptomatic (S) and naturally infected asymptomatic (A) cats, experimentally infected cats (E).
Development of lymphopenia

All 5 of the naturally infected cats with clinical symptoms (Group S), showed lymphopenia (<1.5 x 10^12 lymphocytes/L blood) at the time of sampling. No lymphopenia was found in the naturally FIV-infected cats without clinical symptoms (Group A). Of the experimentally infected cats, only the two animals that were infected with the Petaluma strain showed lymphopenia, developing within four months after infection (154), and which persisted throughout the two years of observation. Similar lymphopenia was never observed in the cats experimentally infected with the Dutch FIV strain during a followup period of 2 years. All the mitogen- and IL-2-induced proliferative responses and the ConA-induced IL-2 production of the PBMC of these two cats were significantly lower than those of the nonlymphopenic animals in Group E.

Discussion

We have studied in vitro parameters of the feline immune system following natural or experimental infection with FIV. Proliferation responses of PBMC from symptomatic and asymptomatic cats after natural or experimental infection with FIV, induced by activation with mitogens or human IL-2, were significantly lower than those of noninfected cats. Similarly, IL-2 production levels of PBMC from symptomatic and experimentally infected asymptomatic cats were significantly lower, whereas the production of IL-2 by PBMC from asymptomatic naturally infected cats was unchanged. It may be concluded that the immune function of the symptomatic, and also of the asymptomatic FIV seropositive cats studied, was seriously impaired. All of these parameters are generally also reduced in HIV seropositive individuals soon after infection (5,25,42,97,181,207). The data of our studies are in line with earlier findings, showing that alterations in the immune system in FIV seropositive cats parallel those in HIV seropositive individuals as exemplified by changes CD4/CD8 ratios (2,200) and lymph node morphology (W. Jarrett, personal communication).

Since most of the FIV seropositive cats in these studies were privately owned spontaneously infected animals, in which the moment of FIV infection was not known, we could not conclude whether the differences in the responses found between individual animals were related to the duration of infection. The data obtained from the 12 experimentally infected cats that were still asymptomatic (followup time: two years) showed that loss of immune function occurs within, at most, 6 months after infection, before the onset of clinical symptoms. Apparently,
an early sign of immunological dysfunction was a reduced responsiveness to IL-2, since no longitudinal data were available from these animals this could not further be substantiated. The fact that ConA-activated cells of infected cats show a reduced response to exogeneous IL-2 while the stimulation indices of these cells for Groups C, A and E stay the same suggests a reduction of the number of cells responding rather than a reduced response per cell. Further studies are needed to clarify this issue. The development of lymphopenia in two of the experimentally infected cats without apparent clinical symptoms, within four months after experimental infection plus the observation that the proliferative responses and the IL-2 production of the PBMC of these two cats was significantly lower than those of the other cats in this group, indicate that either there are individual differences in susceptibility for the development of malfunction of the immune system upon infection, or that strain differences of FIV play a role in the pathogenicity of the virus.

Since secondary infections play a major role in the pathogenesis of human AIDS, it was decided to study whether differences could be found between the respective groups, with regard to virus infections that are commonly found in cats. The experimentally infected cats (Group E) all remained free from these virus infections during the course of these experiments, as shown by serological screening. Serological studies in the symptomatic (Group S) and asymptomatic (Group A) cats, that had naturally acquired FIV infection, revealed no significant differences between these two groups: as expected, serum antibodies to FPV, FCaV, FHV, FeSFV and FCV were present in the majority of the cats of both groups. None of the cats in this study tested positive for FeLV, an important cofactor in the development of feline AIDS following FIV infection (200).

Although more longitudinal studies are needed to define the factors that determine the clinical course of experimental FIV infection of the cat, it may be concluded on the basis of the data presented, the clinical manifestations, and immunological alterations found in symptomatic cats, that the course of FIV infection in the cat resembles that of HIV infection in man. This underlines the value of the FIV model for the development of intervention strategies for AIDS.

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Chapter 2.2

Gag- And env-Specific Serum Antibodies in Cats
After Natural and Experimental Infection
with Feline Immunodeficiency Virus

G.F. Rimmelzwaan, K.H.J. Siebelink, H. Broos,
G.A. Drost, K. Weijer, R. van Herwijnen
and A.D.M.E. Osterhaus

Abstract

In order to monitor the antibody response to feline immunodeficiency virus (FIV) in cats, following experimental and natural infection, enzyme-linked immunosorbent assays (ELISAs) were developed using recombinant env and gag proteins and p24-specific monoclonal antibodies. It was shown that in experimentally infected cats an env protein-specific antibody response was directly followed by a gag protein-specific response. Furthermore, an ELISA for the detection of env protein-specific serum antibodies proved more sensitive in identifying experimentally and naturally infected cats than ELISAs demonstrating gag protein-specific antibodies. It was concluded that, like in HIV infection of humans, the detection of env protein-specific serum antibodies in addition to gag protein-specific antibodies is not only an important tool in the diagnosis of the infection but also in studies concerning the pathogenesis of the disease.

Introduction

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that was first isolated from domestic cats suffering from clinical signs indicative of an immunodeficiency syndrome (199). The disease in cats is characterized by lymphadenopathy and severe impairment of immune function (2,241,269) accompanied by a decrease of the number of CD4 T lymphocytes (104). FIV has been shown to infect cat macrophages (30), astrocytes (53) and the CD4+ subset of T lymphocytes: a cell tropism similar to that of human immunodeficiency virus (HIV). Thus in many respects FIV infection in cats resembles HIV infection in man and therefore FIV infection should be considered a useful animal model for AIDS in man. The model has been shown suitable to study the potential of certain antiviral compounds (55,215), approaches for experimental vaccination (287) and the pathogenesis of lentivirus induced immunodeficiency (289). For these studies the development of assays to monitor the FIV-specific immune response after FIV infection and immunization with candidate vaccines is of critical importance. Several serological assays which demonstrate antibodies specific for the structural proteins of FIV have been described. These include enzyme-linked immunosorbant assays (ELISAs) based on whole virus preparations and immunofluorescence assays.
Establishment of FIV infection model

(IFA), both of which do not discriminate between antibodies directed against the individual FIV proteins (185,239). Also Western blot and radio immunoprecipitation assays have been described (58,109,153), which are, however, too laborious for routine serological procedures. Recently, virus neutralization assays have been developed, which demonstrate antibodies that may directly be involved in protection from infection or development of disease (271).

With the advent of hybridoma, recombinant DNA and peptide synthesis technologies it has become possible to develop assay systems which can demonstrate antibodies directed to individual proteins or even individual epitopes. In the present paper we describe the comparison of four newly developed ELISAs based on the use of FIV-env and p24 recombinant proteins and p24-specific monoclonal antibodies to monitor FIV-specific antibody development in the sera of naturally and experimentally infected cats.

Materials and Methods

Virus preparations

An FIV isolate (Adam 4) from a Dutch cat seronegative to FeLV and FeSFV, was obtained by cocultivating peripheral blood mononuclear cells (PBMC) with Concanavalin-A and Interleukin-2 stimulated PBMC from a specified pathogen-free (SPF) cat as previously described (240). The FIV Petaluma strain was kindly provided by Dr. N.C. Pedersen (Davis, CA, USA). Crandell feline kidney (CrFK) cells (41) were infected with FIV by cocultivation of CrFK cells with the infected PBMC as described (239) and cultured in Dulbecco's minimal essential medium containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.002 M glutamin and 5% fetal calf serum (FCS). Culture supernatants of the infected (and non-infected) CrFK cells were collected seven to ten days after passage of the cells in fresh medium. The culture supernatants were used as antigens in the CTB-ELISA (see below) after clarification by low speed centrifugation (10 min, 2000 rpm).

For immunization purposes FIV was purified from culture supernatant by density gradient centrifugation. The virus was first pelleted by centrifugation for one hour at 100,000 g at 4°C. The pellet was resuspended in phosphate buffered saline (PBS) and then layered over a discontinuous metrizamide (Nyegaard and Co, Oslo, Norway) gradient. After centrifugation for one hour at 100,000 g at 4°C, virus containing fractions identified by a previously described ELISA (239) were collected.

Concentration and partial purification of FIV antigen from culture supernatant
was also performed by precipitation in polymer based aqueous two phase systems as described for other retroviruses (90-92). Polyethylene glycol 6000 (Merck, Schuckardt, Germany) and Dextran T 500 (Pharmacia, Uppsala, Sweden) were added to the culture supernatants at final concentrations of 7% and 0.2% respectively. The mixture was allowed to equilibrate by gently rocking for 16 hours at 4°C. Two phases were allowed to settle for another 16 hours at 4°C. Bottom and interphase (containing FIV) and top phase were collected separately. In this way a concentration of FIV of about 100-fold was achieved. This antigen was used for Western blot assays (see below).

**Generation and selection of monoclonal antibodies (mAbs)**

A panel of hybridoma's producing mAbs in ELISA reactive with FIV, was generated essentially as previously described for the generation of mAbs against poliovirus (195). Briefly, spleen cells from BALB/c mice, which were subcutaneously immunized with 5 μg gradient purified FIV in Freund's complete adjuvant on day 0 and in Freund's incomplete adjuvant on day 14, were fused with a mouse myeloma cell line after intraperitoneal booster injections on days 28, 29 and 30 with 5 μg of pelleted FIV. Hybridomas producing FIV-specific mAbs were selected by screening culture media in an ELISA. For this ELISA microtiter plates (Costar, High binding RIA/EIA plates) were treated by incubating 50 μl volumes of a PBS solution containing 0.125 mg poly-L-lysine and 10 mg 1-ethyl-3(3-dimethylaminopropyl)-carbodiimidehydrochloride (EDC) (Sigma, St. Louis, USA) per ml, for one hour at room temperature. After each incubation the plates were washed with demineralized water containing 0.05% Tween-80 (Merck, Schuckardt, Germany) (DWT). Fifty μl volumes containing 300 ng of pelleted FIV or control antigen were coated in 0.1 M carbonate buffer, pH 9.6 for 16 hours at 20°C. The plates were blocked with 50 μl volumes of PBS containing 0.05% Tween-80 and 1% bovine serum albumin (BSA) (PBS-TB) for one hour at 37°C. Fifty μl of undiluted culture supernatants were transferred to the wells and incubated for one hour at 37°C. After washing, 50 μl volumes of a horse raddish peroxidase (HRP) conjugated goat anti-mouse IgG (Fc) antibody preparation (Cappel, Cooper Biomedical, Malvern, USA) were added to the wells and incubated for one hour. The plates were washed and 100 μl volumes of substrate solution (0.1 mg/ml tetramethyl benzidin (TMB) and 0.003% H₂O₂ in 0.1 M NaAc buffer, pH 5.5) were added to each well. After incubation for 10 min. at 20°C 100 μl volumes of 2 M H₂SO₄ were added to stop the color reaction. The absorbance at 450 nm (A450) was read in a Titertek Multiscan (Titertek, Flow Laboratories). Hybridomas were selected and single cell cloned twice by micromanipulation. Mouse ascitic fluids
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were produced in BALB/c mice. Immunoglobulins (Ig) were isolated from mouse ascitic fluids by precipitation with ammonium sulphate and affinity chromatography using protein A sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) (234). Ig classes and isotypes were determined in an indirect ELISA with specific anti-Ig conjugates (Zymed Laboratories, CA, USA) and indicated in brackets.

Monoclonal antibodies were conjugated to biotin by incubating the antibody solution, which was dialyzed against 0.1 M NaCO₃, pH 8.3 (1 mg protein per ml) with N-hydroxysuccinimido biotin (Sigma, St. Louis, USA) solubilized in dimethyl sulphoxide (1mg/ml) at a 1:8 (w/w) ratio (biotin:protein) for four hours at room temperature. The conjugates were dialyzed against PBS and stored at -20°C.

**SDS-PAGE and Western blot analysis**

FIV (Adam 4) preparations were denatured in the presence of sodium dodecyl sulphate and 2-mercaptoethanol and the individual viral proteins were separated on polyacrylamide gel (12%) by using the discontinuous buffer system (136). Polypeptides were then electrically transferred to nitrocellulose sheets (270). For immunostaining the nitrocellulose was blocked with PBS-TB for one hour at 20°C and after each incubation washed three times for 10 min.

After incubation with monoclonal antibodies or cat sera for one hour at 20°C, the nitrocellulose was incubated with horse radish peroxidase (HRP) conjugated goat anti-mouse IgG (Fc) or goat anti-cat IgG (Cappel, Cooper Biomedical, Malvern, USA) respectively. Subsequently the nitrocellulose strips were developed with 24 mg tetramethyl benzidin and 80 mg diocetyl sodium sulfosuccinate diluted in 10 ml ethanol supplemented with 30 ml 0.1 M citrate/phosphate buffer pH 5.5 and 25 μl 30% H₂O₂.

**Recombinant FIV proteins**

Recombinant FIV env protein, derived by PCR amplification of DNA from bone marrow cells of a naturally FIV-infected cat (cat Adam 19), was obtained by expression through recombinant vaccinia virus (rVV) vGR657 as described (219). In brief, HeLa spinner cells or BHK spinner cells were infected at a m.o.i. of five with rVV vGR657. After 48 hours, the cells were washed with PBS and lysed in PBS containing 0.5% NP-40 and 0.5% SDS. After centrifugation for 20 min. at 10,000 x g, the supernatant was used as FIV-env antigen in the respective assays.

Recombinant FIV p24 and p17 protein were obtained by expression in *E. coli* as fusion proteins using the pGEX-2T expression plasmid as previously described (213).
Cats and serum samples

Six female SPF cats of about 12 weeks old from a breeding colony (Harlan CPB, Zeist, The Netherlands), which is regularly screened for the absence of viral pathogens, were infected via the intravenous route with about $100 \text{TCID}_{50}$ of one of three different FIV strains. Two (#5 and #6) with the Petaluma strain (kindly provided by Dr. N.C. Pedersen, Davis, CA, USA), two (#11 and #12) with a Dutch isolate (Adam 19) and two (#13 and #14) with the progeny virus of the infectious molecular clone 19K1 that was obtained directly from bone marrow DNA of a naturally infected cat, from which also FIV isolate Adam 19 was obtained (240). Serum samples were collected at regular intervals after infection, while the animals were kept in strict isolation.

Serum samples were also collected from 11 privately owned Dutch cats which were suspected of FIV infection on clinical grounds and positive serology in a commercial test (Petcheck, IDEXX Corporation) and from 26 privately owned Dutch cats without clinical signs.

ELISAs for the detection of FIV-specific antibodies

For the detection of FIV-specific antibodies, four indirect ELISAs were used. These ELISAs differed only in the antigen preparations used for the coating of the ELISA plates (Costar RIA/EIA, High binding). Optimal concentrations of the reagents used were determined by checkerboard titration. The coating procedures of the four ELISAs were as follows:

1. Total virus ELISA (TV-ELISA). For the TV-ELISA, FIV antigen for coating was concentrated by ultracentrifugation from supernatants of infected CrFK cell cultures. After solubilization with 1% w/v Triton X-100 (Merck, Schuchardt, Germany), the antigen was diluted in 0.1 M NaAc buffer, pH 5.5, 50 µl volumes were added per well and the plates were incubated at room temperature for 16 hours. The wells were then emptied and fixed with 4% (w/v) paraformaldehyde for 10 min. at room temperature. After washing the plates were blocked with PBS containing 0.05% Tween-80 and 10% FCS.

2. ELISA based on FIV-specific mAbs (MTV-ELISA). For the MTV-ELISA, microtiter plates were coated with purified mAb 8-21 (see below) in 0.1 M carbonate buffer, pH 9.6. The plates were washed with DWT containing 0.05% Tween-80 after each incubation. After blocking with PBS-TB for one hour at 37°C, 50 µl volumes of culture supernatants of FIV infected (or non-infected) CrFK cells, to which 1% Triton X-100 and 5% NaCl was added, were incubated for one hour at 37°C.

3. ELISA based on recombinant FIV-env protein (rec.env-ELISA). For the
rec.env-ELISA cell lysates of rVV vGR657 (or wild type vaccinia virus) infected RK13, BHK or HeLa cells were coated in 0.1 M NaAc buffer, pH 5.5 in 50 µl volumes for 16 hours at room temperature. The wells were then emptied and fixed with 4% (v/v) paraformaldehyde for 10 min. at room temperature. After washing the plates were blocked with PBS containing 0.05% Tween-80 and 10% FCS.

4. ELISA based on recombinant FIV p24/p17 proteins (rec.gag-ELISA). The rec.gag-ELISA, based on recombinant gag proteins, was purchased from the European Veterinary Laboratory B.V., Amsterdam, The Netherlands (FIV-p24/p17 antibody test kit, cat no. F1002-AB01) and performed essentially as described previously (213).

After coating and blocking of the microtiter plates, 50 µl of twofold serial dilutions of serum samples in PBS-TB supplemented with 5% NaCl were added at a starting dilution of 1:40 or 1:20. Then the plates were incubated for one hour at 37°C. Subsequently 50 µl volumes of a biotin conjugated mouse mAb directed to cat IgG (Sigma, St. Louis, USA) or an HRP conjugated goat anti-cat IgG antibody preparation (Cappel, Cooper Biomedicals, Malvern, USA) were added and the plates were incubated for one hour at 37°C. HRP bound streptavidin (Amersham, Amersham, UK) was allowed to bind to biotin for 30 min. at 37°C. After each of these incubations the plates were washed with DWT and subsequently developed with TMB as described above. OD450 values were determined in a Titertek multiscan. Titres were given as the reciprocals of the dilutions still giving more than three times background values obtained with control antigen coated plates.

Complex trapping blocking ELISA (CTB-ELISA) for the detection of FIV p24-specific antibodies

For the CTB-ELISA microtiter plates were coated with FIV p24-specific mAb 8-21 and blocked with PBS-TB as described for the MTV-ELISA. The plates were washed twice after each incubation with DWT. Twenty-five µl volumes of twofold serial dilutions of cat serum samples, starting at a dilution of 1:5 were added simultaneously with 25 µl of a dilution of culture supernatant of FIV infected CrFK cells giving 75% of maximum binding. All dilutions were prepared in PBS-TB supplemented with 1% Triton X-100 and 5% NaCl. After incubation for two hours at 37°C, remaining binding of FIV antigen was detected using biotin conjugated mAb 2-13 (see below). The combination of mAb 8-21 as capture antibody and mAb 2-13 as conjugated antibody was chosen as it proved to be the most sensitive among those tested with the mAbs generated in these studies. After incubation with biotin conjugated mAb 2-13 for one hour at 37°C, HRP bound streptavidin was allowed to bind to biotin for 30 min. at 37°C. The plates were developed as described above.
Test samples were considered positive if a reduction of 50% or more of the signal was observed.

Results

Selection and characterization of FIV-specific mAbs

Five hybridomas, producing antibodies reactive with FIV antigen as determined in the hybridoma screening ELISA, were obtained from two fusion events. These mAbs were designated 2-11 (IgG1,k), 2-13 (IgG2b,k), 3-20 (IgG2a,k), 4-22 (IgG1,k) and 8-21 (IgG1,k). When tested for their reactivities in Western blot analysis, these five mAbs all reacted with the denatured p24 core protein of FIV (Fig. 1). No reactivity was found with control antigen concentrated and purified in the same way. The specificity of these five mAbs for the FIV gag protein was confirmed by demonstrating their reactivities in the rec.gag-ELISA (OD$_{450}$ >800 per 50 ng Ig) in which their respective isotype control mAbs did not react (data not shown).

![Figure 1. Western blot analysis of FIV-specific MAbs. Viral proteins of two phase system purified FIV (lanes 1-7) or control antigen (lanes 8 and 9) were separated by SDS-PAGE and electrically transferred to nitrocellulose. For immunostaining individual lanes were incubated with serum of a FIV seropositive cat (diluted 1:100, lanes 1 and 8), serum of a seronegative cat (diluted 1:100, lane 2), culture supernatants of MAbs 2-11 (lane 3), 2-13 (lane 4), 3-20 (lane 5), 4-22 (lane 6) and 8-21 (lane 7).]

FIV-specific antibody responses in experimentally infected SPF cats

The development of FIV-specific serum antibodies in experimentally infected cats was monitored in the respective ELISAs (Fig. 2). In two SPF cats (#11 and #12) infected intravenously with the Dutch FIV isolate Adam 19 and two other SPF cats (#13 and #14) infected with the progeny of infectious molecular clone (Adam 19K1), the development of serum antibodies to whole virus, FIV env and FIV gag proteins followed similar kinetics (Fig. 2a). Immediately after the onset of env-
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Figure 2. Development of FIV-specific antibodies in experimentally infected cats. Antibody responses were monitored in the TV-ELISA (■-■), MTV-ELISA (□-□), rec.env-ELISA (○-○), rec.gag-ELISA (△-△) and CTB-ELISA (○-○) of: cats infected with the Dutch isolate Adam 19 of FIV (cats #11 and #12), cats infected with the progeny of infectious molecular clone Adam 19K1 (cats #13 and #14) and cats infected with the Petaluma strain of FIV (cats #5 and #6).

Specific response detected in the rec.env-ELISA which was found within three weeks post infection and led to titres >5120 within five weeks, FIV gag protein-specific antibodies, measured in the rec.gag-ELISA and in the CTB-ELISA were detected. These increased rapidly during the subsequent weeks. In cat #13 the antibody response to FIV gag protein seemed to develop slightly later. Antibodies
measured in the TV- and MTV-ELISAs developed concomittently with the env-specific antibodies in these four cats. A relatively late development of FIV gag protein-specific serum antibodies was more pronounced in the two SPF cats (#5 and #6) infected intravenously with the CrFK-cell adapted Petaluma strain of FIV (Fig. 2b). In cat #5 no FIV gag protein-specific antibodies could be detected within eight weeks post infection, whereas in cat #6 FIV gag protein-specific antibody titres slowly increased from five weeks post infection onward. These data coincided with the development of FIV gag protein-specific antibodies detectable in Western blot analysis in these two cats (data not shown). The development of env-specific serum antibodies did exhibit kinetics similar to those observed in the other four cats. Serum antibodies measured in the TV- and MTV-ELISAs in cat #6 were also similar to those of the other four cats, whereas the development of these antibodies in cat #5 again proved to be slower.

**FIV-specific antibodies in clinically healthy and clinically suspected cats**

Privately owned Dutch cats (n=37) with (n=11; positive in the TV-ELISA; Petcheck, IDEXX Corporation) and without (n=26; negative in the TV-ELISA) clinical signs indicative for FIV infection, were tested for the presence of FIV-specific serum antibodies in the four newly developed ELISAs: MTV-, rec.env-, rec.gag- and CTB-ELISAs (Fig. 3).

None of the serum samples from cats without clinical signs scored positive in any of the newly developed ELISAs. The 11 cats with clinical signs indicative for feline AIDS, all scored positive in the rec.env-ELISA, with titres ranging from 320 to ≥5120. Of the 11 positive samples three (samples A, B and C) scored consistently negative in the rec.gag- and the CTB-ELISA, and negative or low titred (titre=20) in the MTV-ELISA. There proved to be an overall relation between the values found with the individual serum samples in the MTV-, rec.gag- and CTB-ELISAs which was not the case when the results of these assays were compared with those obtained in the more sensitive rec.env-ELISA.

**Discussion**

In this paper we have described four newly developed ELISAs based on different principles using recombinant FIV proteins or gag protein-specific monoclonal antibodies. New generations of diagnostic ELISAs are needed since the presently used classical assays, based on whole virus preparations have been shown to score too many false positives (213,239). Furthermore, ELISAs based on this principle do

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Figure 3. Comparison of FIV-specific antibody levels in sera from naturally infected cats (n=11) obtained in the rec.env- and MTV-ELISAs, the rec.env- and rec.gag-ELISAs, the rec.env- and CTB-ELISAs, the rec.gag- and the CTB-ELISAs, the CTB- and the MTV-ELISAs and the rec.gag- and MTV-ELISAs. (For A, B, C: see Results)
not allow the identification of antibodies directed against individual viral proteins, which as in the case of HIV infection of humans may be of major importance for studies concerning the pathogenesis of the disease (7,8,137,281). All except the CTB-ELISA were based on the use of antigen coated plates to quantitate FIV gag- or env-specific antibodies in feline sera. With sera from experimentally infected cats it was shown that, as in HIV-1 infections in man (212,228), env-specific antibodies were detected before the appearance of gag-specific antibodies. This difference was most pronounced in cats infected intravenously with the CrFK adapted Petaluma strain of FIV. The overall slower development of FIV-specific antibodies in these two cats as compared to those infected with the Dutch isolate Adam 19 or the molecular clone 19K1 indicates that either strain differences or adaptation of the virus to CrFK cells may directly influence the initial replication rate of the virus. The relatively slow development of gag-specific antibodies in the cats infected with the Petaluma strain was confirmed by showing that these findings coincided well with the development of antibodies reactive with p24 in the Western blot assay (not shown). After having monitored the development of serum antibodies, detectable with these assays in the experimentally infected cats during the first months after infection, their value for the detection of naturally infected domestic cats was studied by testing the serum samples of 11 cats suspected of suffering from FIV infection on basis of clinical signs and subsequent positive serology in the Petcheck ELISA. All the 26 clinically healthy cats scored negative when tested in all the newly developed serological tests. Among the 11 TV-ELISA positive cats, all scored positive in the rec.env-ELISA. Of these, three (samples A, B and C) were negative or borderline positive in the ELISAs detecting antibodies directed to the FIV gag protein. From cats A and C PBMC could be obtained, from which FIV was isolated (data not shown). This indicates that results found in the rec.env-ELISA are indeed confirmative for FIV infection and that the other three ELISAs may fail to identify some FIV infected cats.

Although the envelope glycoprotein is subject to antigenic variation which may result in lack of reactivity with serum antibodies of cats infected with heterologous virus, no evidence was provided with the serum samples tested that this was the case. However, the combination of serological assays detecting both FIV env- and gag-specific antibodies, circumvents this possible problem and should be considered confirmative for FIV infection.

For the detection of gag-specific antibodies, the use of the MTV-ELISA based on FIV gag-specific monoclonal antibodies may be less suitable since agglomerates of different virus proteins may be bound to the plates. The results obtained with sera from experimentally infected (#5 and #6) cats suggest that this is indeed the
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A discrepancy in the presence of env- vs. gag-specific antibodies, as was shown in the early stages of infection in the experimentally infected cats #5 and #6, may cause false negative serological results when a system is used that does not detect env-specific antibodies. Such discrepant results may perhaps also be expected when cats progress towards AIDS, as has been documented in HIV symptomatic infections in man, in which gag-specific antibodies, either due to immune complex formation or loss of functional B lymphocytes, decline while env-specific antibodies persist (137-139,168,261,281). The discrepancies found in FIV gag- vs. env-specific antibodies in cats A, B and C may be a reflection of this phenomenon since they all exhibited serious signs of immunodeficiency at the time of sampling. Since a small number of animals has been tested, these ELISA systems should be used to test more cats in different stages of disease to assess that these phenomena indeed play a role in FIV pathogenesis. The documented failure of serological assays to identify "silently" FIV infected cats (108), may perhaps be attributed to their inability to detect FIV env protein-specific antibodies. A similar observation has been described for certain HIV infections which initially were believed not to induce serum antibodies (212,228).

In conclusion the data presented indicate that ELISAs based on the simultaneous detection of FIV env and gag protein-specific serum antibodies are highly specific and sensitive tools for the serology of FIV infection in cats. They also allow detailed studies of mechanisms underlying certain kinetics of the FIV-specific antibody response that have been documented also in HIV infections of humans.

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Chapter 2.3

Isolation and Partial Characterization of Infectious Molecular Clones of Feline Immunodeficiency Virus Obtained Directly from Bone Marrow DNA of a Naturally Infected Cat


Abstract

Replication-competent molecular clones of feline immunodeficiency virus (FIV) were isolated directly from the DNA of bone marrow cells of a naturally FIV-infected cat. After transfection in a feline kidney cell line (CrFK) and subsequent cocultivation with peripheral blood mononuclear cells (PBMC), the viral progeny of the clones was infectious for PBMC but not for CrFK cells. PBMC infected with these clones showed syncytium formation, a decrease in cell viability, and gradual loss of CD4+ cells. The restriction maps of these clones differed from those obtained for previously described molecular clones of FIV derived from cats in the United States. The predicted amino acid sequence similarity of the envelope genes of the two clones was 99.3%, whereas the similarities of the sequences of the clones to those of two molecular clones from the United States, Petaluma and PPR, were 86 and 88%, respectively. Most of the differences between the amino acid sequences of the two clones and those of the clones from the United States were found in five different hypervariable (HV) regions, HV-1 through HV-5. The viral progeny of one of these clones was inoculated into two specific-pathogen-free cats. The animals seroconverted, and the virus could be reisolated from their PBMC.

Introduction

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus, initially isolated from an immunodeficient cat (199). FIV infection in cats can lead to immunological abnormalities similar to those seen in human immunodeficiency virus type 1 (HIV-1) infected humans, like a depletion of CD4+ cells in circulation (2,16,269). Similarly, the peripheral blood mononuclear cells (PBMC) from FIV infected cats show reduced proliferative responses to mitogens and to exogenous interleukin 2 (IL-2) in vitro (16,94,241). These similarities in biological behavior between HIV-1 and FIV may make FIV infection of cats a suitable small-animal system to study lentivirus pathogenesis as a model for human AIDS and may likewise identify the FIV system as a model for HIV vaccine development.

Lentiviruses display a large degree of molecular and biological variation. This variation is generally ascribed to the low fidelity of the viral enzyme reverse transcriptase (RT) in copying the viral genomic RNA to DNA (206,220). The use
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of complete molecular clones allows one to obtain genomically homogeneous viral populations. Relating the biological properties of such populations to the genomic structure of the molecular clone from which they were derived may reveal the molecular basis of biological variation and may identify determinants of viral virulence. To date, molecular clones of three FIV strains have been described, two from the United States (the 34TF10 clone of the Petaluma strain [(188,259)] and the PPR clone of the San Diego strain [(204)] and one from Japan (JMI [(173)]), which were all obtained from the DNA of in vitro-propagated cells. Culturing in vitro may select for certain virus subpopulations and can induce new features in the propagated virus. Culturing of simian immunodeficiency virus (SIV) in human cells, e.g., can lead to the introduction of a premature termination codon in the viral transmembrane glycoprotein (102,132). These problems make the availability of complete molecular clones obtained directly from the DNA of in vivo-infected cells highly desirable. Here we report two replication-competent molecular clones derived directly from the DNA of bone marrow cells from an FIV-infected cat. The genomic structure of these clones and their presumed envelope gene sequences are presented.

HIV-1, HIV-2 and SIV infect cells via the CD4 molecule which acts as the cellular receptor for these viruses (45,127,156,166). These viruses are also cytopathic for CD4+ cells in vitro, and this cytopathic effect may at least in part underlie the virally induced immunodeficiency characterized by CD4+ cell depletion in vivo. The CD4 analog in cats has been identified recently (1), but its role in FIV binding and entry is not yet clear. FIV infection also results in a reduction of CD4+ cells in circulation in infected cats. We have therefore investigated whether the molecular clones of FIV described here are cytopathic for CD4+ cells in vitro.

Materials and Methods

Cells and virus

Bone marrow cells were obtained from the femur of a 4-year-old naturally FIV-infected free-roaming cat (Amsterdam-19) suffering from a generally debilitating disease and intermittent disease symptoms, including respiratory infections and chronic diarrhea, all suggestive of an immunodeficiency syndrome (199,289). The cells were washed twice and used for isolation of genomic DNA (see below).

PBMC were isolated from heparinized blood from the cat by Ficoll density gradient centrifugation. The PBMC were washed three times with RPMI 1640 (GIBCO), supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-
glutamin (2 mM), and β-mercaptoethanol (2 x 10⁻⁵ M), designated culture medium (CM). A total of 10⁷ PBMC were stimulated with 5 ug concanavalin A (ConA) (Flow Laboratories, Inc) per ml in CM supplemented with 10% heat-inactivated fetal calf serum (FCS). After 3 days, the cells were washed once and further cultured with CM supplemented with 10% FCS and IL-2 (100 IU/ml) (Cetus). The culture supernatant was monitored weekly for the presence of FIV antigen in an FIV antigen capture enzyme linked immunosorbent assay (ELISA) as described previously (239). A virus stock of FIV from cat Amsterdam-19 (designated FIV Amsterdam-19) was made from the supernatant of this culture 21 days after stimulation and stored in aliquots at -135 °C. The RT activity was assayed and was 7.0 x 10⁴ cpm/ml.

PBMC were derived from heparinized blood of a specific-pathogen-free (SPF) cat by Ficoll density gradient centrifugation. The cells were washed twice and frozen at -135 °C in aliquots in CM supplemented with 10% FCS and 10% dimethyl sulfoxide. Before use, the cells were thawed and stimulated with ConA (5 ug/ml) for 3 days and further cultured with IL-2 (100 IU/ml).

An FIV-susceptible clone of the Crandell feline kidney cell line (CrFK) was obtained from N. Pedersen (289). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin (100 IU/ml), streptomycin (100 ug/ml), L-glutamin (2 mM), β-mercaptoethanol (2 x 10⁻⁵ M), and 10% FCS.

**DNA isolation**

Genomic DNA was isolated from bone marrow cells. A total of 10⁸ bone marrow cells of cat Amsterdam-19 were washed twice and lysed with 100 ug of proteinase K per ml and 0.5% sodium dodecyl sulfate (SDS) for 16 hours at 42 °C. Cesium chloride (1.25 g/ml of lysate) was added, and after centrifugation for 44 hours at 60,000 rpm in a 70Ti rotor (Beckman), fractions containing the high-molecular-weight DNA were collected, pooled, and dialysed against TE (10 mM Tris, pH 8.0, and 0.1 mM EDTA).

**Southern blot analysis of genomic DNA**

Genomic DNA was digested with BamHI or, NheI. After electrophoresis the DNA was transferred to nitrocellulose and hybridized with ³²P-labeled gag probe P2 (see below) in hybridization buffer (3 x SSC [1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM Tris, 5 x Denhardt’s solution, 0.5% SDS, 5 mM EDTA, 50% formamide, 10% dextran sulfate, and 10 ug of salmon sperm DNA per ml). The blots were washed twice with 0.2x SSC - 0.1% SDS for 30 minutes at 68 °C each time and autoradiographed.
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Molecular cloning of FIV proviral DNA

Genomic DNA from bone marrow cells from cat Amsterdam-19 was partially digested with Sau3A. After size fractionation on a 5 to 25% NaCl gradient, 10- to 20-kb fragments were ligated to EMBL-3 lambda arms (Stratagene) and packaged in vitro. The unamplified lambda phage library was screened with the probes P2 (1.8 kb SstI - BamHI gag fragment) and P22 (2.1 kb BamHI - BamHI Pol fragment), which were obtained from R. Olmsted (188) and ENV-4 (a 2.6-kb fragment spanning the complete envelope gene obtained by polymerase chain reaction [216]).

Positive plaques were purified, and the DNA was isolated. SphI - SalI fragments of these clones were subcloned in pUC19. The subclones were used for the dideoxynucleotide chain termination sequence reaction (229) of the env gene. The nucleotide and protein alignments as well as the percentage of similarity were determined with Lasergene software (DNASTAR Inc., London, United Kingdom).

Purified lambda DNA was used for the construction of a restriction enzyme map. Lambda DNA from the clones was digested with the restriction enzymes BamHI, BglII, EcoRI, KpnI, Nhel, SphI, and SstI and after electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose. The filters were subsequently hybridized with P2, P22 or ENV-4 and autoradiographed.

DNA transfection

A sample of 5 ug lambda DNA was transfected into CrFK cells by using the cationic lipid DOTMA (lipofectin; Bethesda Research Laboratories, Inc.) according to the protocol of the manufacturer. Briefly, 5 ug of DNA in 50 ul of H2O was mixed with 50 ul lipofectin and incubated for 15 minutes at room temperature. CrFK cells were washed with serum-free Dulbecco’s modified Eagle’s medium, and the lipofectin reagent - DNA complex was added. Twenty-four hours after transfection CM containing 20% FCS, 100 IU of IL-2 per ml, and 5 x 10^6 ConA- and IL-2 stimulated PBMC from an SPF cat were added. After 72 h, the PBMC and CrFK cells were washed and cultured separately. The culture supernatant was monitored for the presence of FIV antigen in an FIV antigen capture ELISA. Eighteen days after transfection, a virus stock was made and stored in aliquots at -135°C. The RT activity was determined. The infected cells were used for electron microscopy essential as described by Gelderblom et al. (78).

Infection of PBMC

ConA and IL-2 stimulated PBMC of an SPF cat were infected with the molecular clones or with the biological isolate FIV Amsterdam-19 by resuspending...
$10^7$ PBMC in 1 ml of virus dilution in CM containing $2 \times 10^4$ cpm of RT activity per ml. After 1 h at 37 °C, the cells were washed twice with CM and cultured in CM supplemented with 10% FCS and 100 IU of IL-2 per ml. Twice a week, 1.5 ml of culture supernatant was centrifuged (10 min at 300 x g) and stored at -135 °C until testing for presence of FIV antigen and RT activity. The cells were monitored for cytopathic effects like syncytium formation and cell viability and for the percentage of CD4+ and CD8+ cells by fluorescence-activated cell sorter analysis with monoclonal antibodies provided by M. Cooper (1,129).

**RT assay**

RT activity was assayed in a microassay as previously described by Gregersen et al (87), with minor variations. Briefly, 1 ml culture supernatant was precipitated with 0.25 ml 32% polyethylene glycol 6000-1.5 M NaCl. The pellets were resuspended in 10 ul of lysis buffer (50 mM Tris [pH 8.3], 20 mM dithiothreitol, 0.25% Triton X-100) and mixed with 40 ul H2O and 50 ul of RT cocktail (100 mM Tris [pH 7.9], 150 mM KCl, 10 mM MgCl2, 4 mM dithiothreitol, 0.6 U poly(rA) - oligo(dT), 60 uCi of $[^3H]$-TTP per ml). After incubation at 37 °C for 1 h, the DNA was precipitated with 20 ul of 120 mM Na2P2O7 . 10 H2O in 60% trichloroacetic acid for 15 minutes at 4 °C. The DNA was spotted onto glass fiber filters with a Skatron cell harvester and washed with 12 mM PPNa in 5% trichloroacetic acid. The filters were dried, and $[^3H]$ TTP incorporation was measured in a β-scintillation counter.

**Nucleotide sequence accession number**

The sequences reported in this paper have been deposited in the GenBank database (accession no. for clone 19k1 is M73964 and for clone 19k32 is M73965).

**Results**

**Molecular cloning and characterization of FIV provirus**

Genomic DNA from bone marrow cells of a naturally FIV-infected free-roaming cat (Amsterdam-19) was digested with restriction enzymes and blotted onto nitrocellulose for hybridization with probe P2, FIV-specific could be detected in this DNA preparation as well as in the DNA obtained from the spleen cells of cat Amsterdam-19 and the spleen and bone marrow cells of another naturally FIV-infected cat (data not shown). Digestion with BamHI or NheI yielded internal FIV fragments with sizes of 3.8 and 3.2 kb respectively. These fragments are also found
in the maps of lambda clones 19k1 and 19k32 (see below).

Full length proviral FIV clones were obtained from a EMBL-3 lambda phage library made directly from the DNA of the bone marrow cells of cat Amsterdam-19 by using the P2, P22, and ENV-4 probes for screening. The hybridizing clones were plaque purified and used for the isolation of lambda DNA. The replication competence of the cloned FIV provirus was assayed by transfection into CrFK cells (see below). Three clones (19k1, 19k32, and 19k36) gave rise to FIV antigen production upon transfection and were characterized further. Restriction maps of these three clones showed a high degree of similarity (Fig. 1). The maps of clones 19k1 and 19k36 were identical, whereas an additional BglII site is present in the envelope gene of 19k32. This additional BglII site was later confirmed by sequence analysis. When the predicted maps of the two U.S. clones (204,259) were compared with that of clone 19k1, only 14 of 22 (34TF10 compared with 19k1, respectively) and 14 of 21 (PPR compared with 19k1, respectively) restriction sites appeared to be conserved. Between 34TF10 and PPR, 12 of 21 restriction sites are conserved (Fig. 1). This indicates a high degree of heterogeneity among these different FIV strains.

![Restriction map of infectious clones molecular clones of the FIV strains Petaluma (34TF10), San Diego (PPR) and FIV Amsterdam-19 (19k1 and 19k32). Restriction enzyme maps of 34TF10 and PPR are as predicted from their published nucleotide sequences (24, 30). Maps of 19k1 and 19k32 were obtained by single and double restriction enzyme digestion of lambda DNA which contain the full length clones, followed by Southern blotting and hybridization with the ³²P labeled FIV specific probes P2, P22, or ENV-4. Restriction enzyme cleaving sites: B, BamHI; Bg, BglII; RI, EcoRI; K, Kpol; N, Nhel; Sp, SphI and S, SstI.](image)
We obtained the nucleotide sequence of the envelope gene of the three clones of FIV Amsterdam-19. The nucleotide sequences of the envelope genes and the preliminary restriction site analysis of the 5' and 3' flanking sequences of the clones 19k1 and 19k36 showed no differences, which suggests that these clones are fully identical. On the other hand, we found that clones 19k1 and 19k32 differed in the length of their flanking sequences (data not shown), and only the sequences of these clones were analyzed further. The 2,571 nucleotide of the envelope genes of these clones differ in only nine positions. Because of the lack of direct amino acid sequence data, it is as yet unclear where the exact initiation codon of the envelope gene is located. We have presumed the conserved ATG at position 6264 of clone 34TF10 of the Petaluma strain (259) to be the initiation codon for the env gene, which then encodes an 857-amino-acid glycoprotein with a potential cleavage site after 611 amino acids, resulting in a 611-amino-acid surface glycoprotein and a 246-amino-acid transmembrane glycoprotein. The predicted amino acid sequences of the envelope glycoproteins of these clones were compared with those of the 34TF10 clone of the Petaluma strain and the PPR clone of the San Diego strain (204,259) (Fig. 2). The amino acid identity of the envelope glycoproteins of clones 19k1 and 19k32 is 99.3%. The sequence identities of these two highly related clones with the 34TF10 and PPR clone envelope sequences are 86 and 84%, respectively. We have aligned the four envelope gene sequences (Fig. 2). Although amino acid variation is found throughout the entire env gene, we observed the presence of five hypervariable (HV) regions, designated HV-1 through HV-5, as indicated in Fig. 2. These HV regions concur in part with the variable regions described by Phillips et al. (204). All four sequences are remarkably colinear, with insertions and/or deletions only found in HV-5 (Fig. 2). Most of the potential N-linked glycosylation sites are conserved between the envelope sequences of all four clones (21 of 22); some variation of the cystein residues is observed within the first 150 amino acids.

**Biological characterization**

Lambda phage, containing apparently full-length proviral DNA of clones 19k1, 19k32, and 19k36, was transfected into CrFK cells. These were then cocultivated with PBMC for 72 hours. Both cell populations were then cultured separately. In the PBMC cultures infected with clone 19k1 and 19k32, FIV antigen could be detected within 8 days after transfection. FIV antigen was also detected in the PBMC culture of clone 19k36 12 days after transfection. Transmission electron microscopy pictures from all of these cultures showed mature and immature virus particles and particles budding from the cell membrane (data not shown).
antigen could not be detected in the supernatant of the CrFK cell cultures. The PBMC cultures were expanded, and virus stocks were stored at -135 °C. The RT activities of the stocks were 2.3 x 10⁴, 2.9 x 10⁴, and 0.5 x 10⁴ cpm/ml for 19k1, 19k32, and 19k36, respectively. On the basis of apparent genetic identity of 19k1 and 19k36 (see above), we have concentrated on clones 19k1 and 19k32 for further analysis.
Chapter 2

Figure 3. Virus production, cell viability, and percent of CD4⁺ cells in feline PBMC infected in vitro with FIV as described in the text. (A) RT activities in culture supernatant; (B) FIV antigen in culture supernatant; (C) percent of viable cells; (D) percentages of viable cells which were CD4⁺. Symbols: *, non-infected; □, biological isolate FIV Amsterdam-19; ■, molecular clone 19k1; +, molecular clone 19k32.

ConA and IL-2- stimulated PBMC (10⁷) from an SPF cat were infected with equal amounts of RT activity (2x10⁴ cpm) of the viral progeny of clone 19k1 or 19k32 or the biological isolate FIV Amsterdam-19 obtained from in vitro-propagated PBMC. The cultures were monitored for syncytium formation, cell death, percentage CD4⁺ and CD8⁺ cells, FIV antigen production, and RT activity. The results are shown in Fig 3. Syncytium formation was seen 4 days postinfection (p.i.) in the culture infected with 19k1 and 6 days p.i in the culture infected with
Establishment of FIV infection model

Figure 4. Syncytium formation of feline PBMC which are infected with viral progeny of molecular clone 19k1 (magnification 400 x).

19k32 or the biological isolate (Fig. 4). FIV antigen and RT activity could be detected after 11 (19k1) and 14 (19k32 and FIV Amsterdam-19) days in the FIV-infected cultures but not in the non-infected control culture for 28 days p.i. (Fig. 3A and B).

The percent viable cells decreased from 95 to 55% during 28 days of culturing in the noninfected culture, whereas there was a more rapid decrease of viable cells in the FIV-infected cultures (only 15% viable cells after 28 days) (Fig. 3C), indicating cell death caused by FIV infection.

The percentages of the viable cells which were CD4+ and CD8+ cells could be measured accurately only during 18 days of culture following infection because of the low percent viable cells after this period. The percentages of viable cells which were CD4+ and CD8+ cells were 44 and 35%, respectively, at the time of infection. After 18 days of culture, these percentages in the non-infected culture were 36 and 38%, respectively. The mean percent CD4+ cells in all of the infected cultures decreased to 6% in 18 days p.i.. The mean percent CD8+ cells increased to 66% in this period (data not shown), indicating a selective depletion of CD4+ cells in infected cultures (Fig. 3D).

Two SPF cats were infected with the viral progeny of molecular clone 19k1, and two cats were infected with the biological isolate FIV Amsterdam-19. FIV could be
isolated from these cats 2 weeks p.i., and seroconversion occurred 2 weeks later (data not shown). This demonstrates that these viruses are infectious in vivo.

Discussion

We have generated molecular clones of FIV directly from the DNA of bone marrow cells of an FIV-infected cat and characterized these clones for genetic and biological properties. Cloning directly from in vivo-infected cells obviates the need for in vitro culturing of FIV in cell lines or in stimulated PBMC. In vitro culture systems may select some viral genotypes over others, as has been shown in numerous instances for HIV-1 (44,66,171), or may even introduce modifications in the cultured virus as demonstrated for SIVmac (102,132). These drawbacks bring the inherent danger that a molecularly cloned virus obtained from in vitro cultures is not representative of the virus population present in vivo in the infected animal. The high load of FIV proviral DNA in the bone marrow cells of the naturally infected symptomatic cat Amsterdam-19 has allowed us to obtain complete molecular clones of FIV directly ex vivo. FIV specific DNA could be detected in genomic DNA of bone marrow cells of cat Amsterdam-19 and in the DNA of bone marrow cells of only one of six other naturally infected cats. It is as yet unclear whether the detection of FIV proviral DNA in the genomic DNA of bone marrow cells of FIV-infected cats is a general feature. In this paper, we show that the viral progeny of these clones has biological properties very similar to the virus isolate obtained from the PBMC of cat Amsterdam-19.

FIV has been shown to cause a depletion of CD4-bearing cells in infected animals, as HIV-1 does in infected humans (2,16,269). For HIV-1, this depletion may be partly explained by its selective tropism for and cytopathic effect on CD4+ cells, which has been demonstrated in vivo. Additionally, HIV-1 can downregulate the CD4 receptor on infected cells (38,43). Although the CD4 analog in cats has been identified, the results as to its functioning as the receptor for FIV have so far been inconclusive. We demonstrate here that an FIV isolate as well as two infectious molecular clones of FIV selectively deplete CD4+ cells from a culture of feline PBMC (Fig. 3 D). The viruses also induce the formation of syncytia in these cultures, indicating that the observed CD4+ cell depletion could well be the result of direct cytopathic effects, although we cannot rule out that downregulation of CD4 also plays a role. The fact that these molecularly cloned viruses induce syncytia formation and deplete CD4+ cells in vitro may indicate that they do the same in vivo upon experimental infection. Such effects would result in progression towards
Establishment of FIV infection model

immunodeficiency. In preliminary experiments, the viral progeny of at least one of the two described clones (namely, 19k1) proved to be infectious in vivo. The experimentally infected animals will monitored closely for hematologic and immunologic parameters as well as for signs of FIV disease.

To facilitate transfection of the molecular clones, we have relied on an adherent cell line that is permissive for FIV replication, namely, the CrFK cell line. After transfection, the CrFK cells produced FIV particles whose viral progeny was thereupon rescued by cocultivation with feline PBMC. Interestingly, we could not show productive reinfection of CrFK cells by these viruses, indicating that one round of replication is not sufficient for adaptation of FIV to CrFK cells. At the same time, these data demonstrate that the lack of growth of many FIV strains on CrFK cells is the result of a block at viral entry rather than at virus replication.

To date, two envelope sequences of FIV molecular clones have been obtained. The comparison of the sequences of these two U.S. isolates revealed an amino acid sequence similarity of approximately 85%. To assess the amount of variation between strains from widely different geographical locations, we have obtained the complete envelope sequence of the two molecular clones 19k1 and 19k32. These two sequences are remarkably similar (99.3% similarity), whereas they differ by approximately 15% from both U.S. isolates, which is the amount of variation found between the U.S. isolates. A high degree of sequence similarity between two different proviruses is unusual for lentiviruses, which in general show much more sequence variation, especially in the envelope gene. The observed conservation may indicate that the obtained FIV clones are the progeny of the original viral clone that colonized the bone marrow of cat Amsterdam-19.

To predict the amino acid sequence of the envelope gene, we took the ATG at position 6264 of the 34TF10 clone of the Petaluma strain to be the start codon for env. This results in an 857-amino-acid glycoprotein with a potential cleavage site at position 611 (259). The precursor of 857 amino acids can be cleaved into two smaller glycoproteins: an outer membrane protein of 611 amino acids and a transmembrane protein (TM) of 246 amino acids. The newly generated N terminus at the presumed transmembrane glycoprotein is highly hydrophobic and could serve as a fusion domain as demonstrated for the homologous regions in SIVmac and HIV-1 (27,74). Although there is no obvious sequence similarity between this domain in FIV and those of SIV and HIV-1, we observed a high incidence of amino acids with very short side chains like glycine and alanine in all three viruses. This GA repeat may well be important for the function of the fusion domain. Most of the structurally important features of the envelope glycoproteins, like cystein residues and N-linked glycosylation sites, are conserved between these clones. Some
nonconserved cysteine residues were found in the putative L gene region of the envelope glycoprotein, preceding the leader sequence identified by others (204,259).

The molecular clones described in this report have shown to be infectious both \textit{in vitro} and \textit{in vivo}. Such clones may enable us to delineate the molecular basis of the pathogenesis of FIV.

\textbf{Acknowledgements}

We are grateful to R. Olmsted for kindly supplying us with the \textit{P}$_{2}$ and \textit{P}$_{22}$ cDNA probes, to N. Pedersen for the CrFK cells and to M. Cooper for providing the CD4- and CD8-specific monoclonal antibodies. We acknowledge K. Teppema and M. Burger for performing the electron microscopy and J. Sonsma for performing the RT assay. We thank C. Kruyssen and M. Eskens for preparing the manuscript.

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Chapter 3

FIV Envelope Determinants Involved in Cell Tropism and Escape from Neutralizing Antibodies
A Determinant of Feline Immunodeficiency Virus Involved in Crandell Feline Kidney Cell Tropism

Kees H.J. Siebelink, Jos A. Karlas, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus, and Marnix L. Bosch

Abstract

Viral progeny of the molecular clone 19k1 of feline immunodeficiency virus (FIV) can infect feline T-cells but not Crandell feline kidney (CrFK) cells. In contrast, the biological isolate FIV-AM6c, which was CrFK adapted by co-cultivation of FIV-AM6 infected thymocytes with CrFK cells, can infect both thymocytes and CrFK cells. The envelope gene of FIV-AM6c was amplified by polymerase chain reaction using DNA from infected CrFK cells, and subsequently cloned and sequenced. To map viral determinants of CrFK cell tropism, chimeric viruses with a 19k1 background containing envelope gene fragments of FIV-AM6c were constructed. CrFK cells were transfected with DNA of these chimeric clones and co-cultivated with thymocytes. After 3 days the CrFK cells and the thymocytes were cultured separately. FIV antigen could be detected in most of the thymocyte cultures within 14 days and in one of the CrFK cultures after 52 days. The resulting virus from this CrFK culture can infect both CrFK cells and thymocytes. The results of this study indicate that the envelope region contains determinants of CrFK tropism. The delay in replication indicates that also determinants other than those identified here are involved in CrFK cell tropism. More chimeric clones are being studied at present to map these determinants.

Introduction

Feline immunodeficiency virus (FIV), like the other lentiviruses, displays a high degree of sequence variation in the envelope gene. Most of this variation maps to the so-called variable regions (197,204). In other lentivirus systems, this genetic variation often has biological consequences, e.g. with regard to cytopathic potential and cell tropism (37). The variable region number 3 (V3) of the human immunodeficiency virus type 1 (HIV-1) envelope, which contains the principal neutralizing determinant, also contains the primary determinants for both cell tropism and for syncytium inducing capacity (88), although variation in both these parameters has also been mapped to regions other than V3 (10,264).

FIV can infect a variety of cell types, like CD4 and CD8 positive T-cells, macrophages and astrocytes (29,30,53). Furthermore some strains of FIV can infect Crandell feline kidney cells (CrFK cells) upon adaptation in vitro (204). Infection
of CrFK cells with FIV is widely used in virus neutralization assays. Using this system a variety of FIV neutralizing antibodies have been described. Most sera that neutralize FIV infection of CrFK cells tend to be broadly reactive, i.e. they all neutralize the different CrFK-adapted FIV strains, independent of the viral isolate against which they were initially raised (272). Such FIV neutralizing antibodies have also been generated in rabbits and mice using a synthetic peptide corresponding to the V3 region of the FIV envelope (148). This peptide also corresponds to an immunodominant epitope in FIV infected cats (13), which suggests that antibodies to this region contribute to the FIV-neutralizing activity in the sera of these cats.

Recently we have described a virus neutralization assay for FIV using activated T cells, one of the natural target cells for the virus (242). With this assay we have only been able to demonstrate genotypic-specific virus neutralization: single point mutations, either induced in vitro, or naturally occurring, completely abolished virus neutralization by a polyclonal serum. No evidence for broad cross-neutralization of FIV was found. These differences in virus neutralization between the two systems, led us to investigate the molecular basis for the adaptation of FIV to CrFK cells. Presently nothing is known of the relationship between genetic variation of FIV and cell tropism. Information regarding this issue may help us to understand the requirements for the infection of different cell types by FIV and, at the same time, may reveal the important biological aspects involved in virus neutralization in both systems.

In order to determine whether the adaptation of FIV to CrFK cells is dependent on virus entry we have exchanged parts of the surface glycoprotein of FIV molecular clone 19k1 (which cannot replicate in CrFK cells) with the homologous fragments of the CrFK-adapted virus strain FIV-AM6c. We examined these chimeric clones for the potential to replicate in feline thymocytes and/or CrFK cells.

Materials and Methods

Cells and virus

Peripheral blood mononuclear cells (PBMCs) were derived from heparinized blood of a 2-year-old specified pathogen-free (SPF) cat by Ficoll density gradient centrifugation. Thymocytes were derived by homogenizing the thymus of an 8-week-old SPF cat. PBMCs and thymocytes were washed twice and frozen at -135 °C in aliquots. Before use the cells were thawed and stimulated with Concanavalin A (Con A) (5 µg/ml) in CM [RPMI-1640 supplemented with penicillin (100
IU/ml), streptomycin (100 µg/ml), L-glutamin (2 mM) and β-mercaptoethanol (2 x 10⁻⁵ M) and 10% fetal calf serum). After 3 days the cells were washed and cultured in CM supplemented with IL-2 (100 IU/ml).

An FIV susceptible clone of the CrFK cell line was obtained from N. Pedersen (289) and cultured as described previously (240).

Replication competent molecular clone 19k1 was obtained directly from bone marrow cells of a naturally FIV infected cat as described previously (240).

FIV was isolated from PBMCs from 11 naturally FIV infected cats. To this end the PBMCs of these cats were stimulated with Con A. After 3 days the cells were washed and co-cultured with Con A and IL-2 stimulated PBMCs from a SPF cat in CM supplemented with IL-2 (100 IU/ml). The culture supernatant was tested weekly for the presence of FIV antigen by ELISA (see below). When FIV antigen was detected subconfluent monolayers of CrFK cells were co-cultivated for one week with 10⁶ infected PBMCs. The CrFK cells were washed, trypsinized and subcultured at a split ratio of 1:5 weekly. The culture supernatants were tested weekly in an FIV antigen ELISA. One of the FIV isolates which was adapted to replicate in CrFK cells was designated FIV-AM6c and was used in this study.

FIV antigen ELISA

Culture supernatants were tested in an ELISA for the presence of FIV antigen as described previously (239).

Generation of chimeric clones

The envelope gene of FIV-AM6c was amplified by polymerase chain reaction (PCR) as described previously (242). The env gene was cloned into pUC19 and the sequence was determined by the dideoxynucleotide chain termination reaction. The nucleotide sequence was compared with the sequence of the envelope gene of molecular clone 19k1. To generate chimeric clones four conserved restriction sites were used to exchange parts of the envelope gene of 19k1 with corresponding parts of FIV-AM6c (Fig. 1). Three chimeric clones were generated: 19k1PBAM6 (19k1 which contain the 144 bp PflMl-Bsal fragment of FIV-AM6c), 19k1BNAM6 (19k1 which contain the 343 bp Bsal-Nsil fragment of FIV-AM6c) and 19k1NKAM6 (19k1 which contain the 248 bp Nsil-Kpnl fragment of FIV-AM6c).

Transfection

Five micrograms DNA of the molecular clone 19k1 and the chimeric clones 19k1PBAM6, 19k1 BNAM6 and 19k1NKAM6 were transfected into CrFK cells using the cationic lipid DOTMA (lipofectin; Bethesda Research Laboratories Inc.,)
Envelope protein determinants

Figure 1. Schematic representation of the envelope genes of molecular clone 19k1 and the chimeric clones of 19k1 which contain the hypervariable regions 3, 4 or 5 of the CrFK adapted isolate FIV AM6c. The uppermost bars represent the major open reading frames gag, pol and env. The black boxes represent the hypervariable regions in the envelope protein. The patterns are corresponding to the parental clones.

according to the protocol of the manufacturer. After 24 h the transfected cells were cocultivated with Con A- and IL-2-stimulated feline thymocytes. After 3 days the CrFK cells and thymocytes were cultured separately. The culture supernatants were monitored for the presence of FIV antigen by ELISA weekly. When FIV antigen could be detected, the culture supernatant was collected, filtered through a 220 nm-pore-size filter and stored in aliquots.

Results

Adaptation of FIV to replicate in CrFK cells

Con A and IL-2 stimulated PBMCs were infected with 11 FIV isolates in separate cultures. Within 7 days FIV antigen could be detected in the supernatants of all cultures by ELISA (Table 1). The PBMCs were then co-cultured with CrFK
Chapter 3

Table 1. FIV antigen detection in culture supernatants of PBMC's and CrFK cells

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NT: Not tested

cells. In four cultures a cytopathic effect (cpe) typical for feline syncytium forming virus (FeSFV) was observed in the CrFK cell culture (not shown). These cultures were discarded. FIV antigen could only be demonstrated in the CrFK cell culture infected with FIV-AM4 and FIV-AM6 28 days post infection (Table 1). Viral progeny of molecular clone 19k1 could not be adapted to replicate in CrFK cells using this procedure (not shown).

Transfection of chimeric clones

Upon transfection with DNA of the molecular clone 19k1 and the chimeric clones 19k1PBAM6, 19k1BNAM6 and 19k1NKAM6 the CrFK cells were cocultivated with thymocytes. After 72 h the CrFK cells and thymocytes were cultured separately and monitored for the presence of FIV antigen in the culture supernatant. Within 3 weeks post transfection FIV antigen was detected in the supernatant of all the thymocyte cultures (Fig. 2a). Only in the culture supernatant of the CrFK cells, which were transfected with 19k1PBAM6 FIV antigen could be detected 52 days post transfection whereas no antigen could be detected in the culture supernatant of the CrFK cells, which were transfected with 19k1, 19k1BNAM6 or 19k1NKAM6 (Fig. 2b).

The culture supernatant of the CrFK cells, which was found positive in the FIV
Envelope protein determinants

antigen ELISA was collected 52 days post transfection, filtered and designated FIV-19k1PBAM6c. CrFK cells were infected with FIV-19k1PBAM6c or FIV-AM6c and monitored at regular intervals for FIV antigen production in the culture supernatant. Within 11 days FIV antigen could be detected in the supernatant of both cultures (Fig. 3). These results show that chimeric clone 19k1PBAM6c is adapted to infect and replicate in CrFK cells with the same kinetics as FIV-AM6c.

Figure 2. FIV antigen production in culture supernatant of PBMC’s (A) and CrFK cells (B) after transfection with molecular clone 19k1 (●) and the chimeric clones 19k1PBAM6 (■), 19k1BNAM6 (▲) and 19k1NKAM6 (○).
Figure 3. FIV antigen production in culture supernatant of CrFK cells infected with FIV-AM6c (□) and FIV-19k1PBAM6c (■).

Sequence analysis

To minimize additional mutations by long term culture, 4 days after infection with 19k1PBAM6c, CrFK cells were lysed and the FIV envelope gene was amplified by PCR and cloned into pUC19. The surface protein was sequenced and compared with the sequence of 19k1. The 19k1PBAM6c sequence differs at five positions from the 19k1 env sequence (Fig. 4). Four of these, three situated in HV-3 and one between HV-3 and HV-4, are the result of the exchange of the 144 bp PflMI-Bsal fragment of FIV-AM6c. An additional G to A mutation at nucleotide position 1225 was observed, which causes an amino acid substitution mutation of glutamic acid to lysine at position 409.

--- HV-3 ---

--- PflMI - Bsal fragment ---

19k1
CGWNQKAYYNQCSWEGTDVKFOCQRTQSGSWIRAISSWRQRNRWRPDESER
19k1PBAM6c
.....Y.....S.R.........................T.........................K.

Figure 4. Amino acid sequence of 19k1 and 19k1PBAM6c from amino acid position 354 to 8410. The 144 bp PflMI - Bsal fragment and the HV-3 region is indicated at the top.
Envelope protein determinants

Discussion

A limited number of all FIV isolates known to date replicate in CrFK cells. These cells are widely used in FIV-neutralization assays which makes elucidation of the molecular basis for CrFK-adaptation of FIV an important research goal. This importance is underlined by the fact that using these cells predominantly broadly neutralizing antibodies are detected, which contrast with the results using T cells, one of the natural target cells for FIV.

The viral progeny of FIV molecular clone 19k1 cannot infect CrFK cells, and we have never been able to adapt FIV 19k1 to grow in these cells even after prolonged periods of cocultivation of CrFK cells with FIV 19k1 infected T cells (up to 3 months). Transfer of an envelope fragment of the CrFK-adapted FIV-AM6c isolate to 19k1 has now allowed us to adapt 19k1 to CrFK cells after 52 days. This envelope fragment contains the variable V3 region; no other envelope fragments have yielded similar results. The resulting adapted virus is now able to infect CrFK cells and does not demonstrate the 52-day delay observed in the adaptation process. It is therefore likely that the fully adapted virus has accumulated other mutations, in the envelope gene or elsewhere, that have now converted the phenotype of the virus to the fully adapted form. One of such changes, just 3' of the originally exchanged V3 region is discussed below. Preliminary experiments indicate that this single mutation is not by itself sufficient for adaptation (in preparation). We currently investigate the contribution of other envelope regions and of the other viral genes to CrFK adaptation.

Other lentiviruses, like HIV-1, also display a high degree of genetic variation in the envelope gene. Mapping experiments have demonstrated that the highly variable V3 region of the HIV-1 envelope contains an important determinant for cytopathicity and cell tropism, as well as the principal neutralization domain (88). In our experiments we could only adapt FIV 19k1 to replicate in CrFK cells when we replaced the V3 region of 19k1 with the envelope V3 region of the CrFK-adapted virus FIV-AM6c, suggesting that here also the V3 region plays a role in determining cell tropism. This parallel between the functional roles of the V3 regions of HIV-1 and FIV can be extended when we take into account that a synthetic V3 peptide of both of these viruses can elicit virus neutralizing antibodies. Interestingly in the FIV system these antibodies will only neutralize FIV infection of CrFK cells, and not of primary T cells. Understanding the requirements for FIV to productively infect CrFK cells may elucidate the basis for virus neutralization in both systems. This information will prove useful in the rational design of virus vaccines that aim at inducing high titers of broadly reactive virus neutralizing
antibodies, both for FIV and for HIV-1.

Since we have determined only the sequence of the gene encoding the surface glycoprotein in this study, we cannot exclude that other mechanisms play a role in the acquisition of CrFK cell tropism by FIV. Our present studies focus on the potential involvement of the gag, pol and regulatory genes of FIV in this phenomenon.

Acknowledgements

We thank C. Kruysen for preparing the manuscript and W. Puyk for performing the pepscan analysis. This work was supported by the Advisory Council on Health Research (RGO No. 88-90/89028) and the concerted action on feline AIDS of the EC.
A Single Amino Acid Substitution in Hypervariable Region 5 of the Envelope Protein of Feline Immunodeficiency Virus Allows Escape from Virus Neutralization

Kees H.J. Siebelink, Guus F. Rimmelzwaan, Marnix L. Bosch, Rob. H. Meloen, and Albert D.M.E. Osterhaus

Abstract

We infected a specific-pathogen-free cat (cat 14) with molecularly cloned feline immunodeficiency virus clone 19k1 (19k1 [K.H.J. Siebelink, I. Chu, G.F. Rimmelzwaan, K. Weijer, A.D.M.E. Osterhaus, and M.L. Bosch, J.Virol. 66:1091-1097, 1992]). Serum of this cat obtained 22 weeks post infection (serum 1422) neutralized FIV19k1 but not FIV 19k32, which is 99.3% identical to FIV19k1 in the envelope gene. Serum 1422 also neutralized virus isolated from cat 14 at weeks 2 and 32 postinfection. We then cultured FIV19k1 in the continuous presence of serum 1422, which resulted in a delay in virus replication of 6 weeks. The resulting virus population appeared to be resistant to virus neutralization by serum 1422. Nucleotide sequencing of the env open reading frame of this presumed escape mutant revealed the presence of one silent and two substitution mutations, both of the latter in hypervariable region 5. Through the construction of chimeric viruses and site-directed mutagenesis, we demonstrated that one of these mutations, the substitution of lysine to glutamine at amino acid position 560 in hypervariable region 5, was sufficient to allow the escape of FIV19k1 from neutralization by serum 1422.

Introduction

Feline immunodeficiency virus (FIV) is a recently discovered T-lymphotropic lentivirus (199) which appeared to be the cause of an AIDS-like disease in naturally infected cats (27,108,199,236,248,289). FIV resembles human immunodeficiency virus type 1 (HIV-1) in its biological and morphological properties. A decline in numbers of CD4+ lymphocytes observed in vivo and in vitro (2,16,104,240), a gradual loss of immune function (16,94,147,241,269,289) and the occurrence of opportunistic infections as in human AIDS make FIV infection in cats a useful small animal model for the study of HIV infections in humans.

Infection with lentiviruses, including FIV and HIV-1, generally results in persistence of the virus in the host, leading to a continuous interaction between the virus and the immune system (67,73,170,176,182,183,289). Genetic variation allows the virus to escape from the continuous pressure exerted by antibodies (169,214,284, for a review, see reference 187) and cytotoxic T cells (202). Domains
on the envelope glycoprotein that induce virus neutralizing (VN) antibodies have been determined (76,85,103,131,264) and escape from VN antibodies has been described in various lentiviral systems. After infection with HIV-1, two types of VN serum antibodies are observed. Shortly after infection, type-specific neutralizing antibodies that are mainly targeted to the principal neutralizing determinant (PND) within the variable region 3 arise (85,196,227). Later in the course of infection, more broadly neutralizing antibodies, in which other antigenic sites on the envelope protein are also involved are induced (76,122,162,208,264; for a review, see reference 84). The envelope gene of FIV is highly variable but exhibits, like the envelope gene of HIV-1, constant and more variable regions (179,204,240). FIV neutralizing antibodies have been demonstrated in the sera of infected cats (69,271), but the sites involved in their induction have not been identified. The generation of VN escape mutants and studies concerning the mechanisms leading to escape may provide more information about the structural and functional properties of these antigenic sites. Two mechanisms by which a lentivirus can escape from VN antibody pressure have been described. The first involves changes in the binding site itself, whereas the other involves changes elsewhere on the envelope glycoproteins (169,284). The availability of molecular clones of HIV-1 has allowed the identification of the molecular basis underlying the escape of the virus from neutralizing antibody and has provided evidence that both mechanisms are operational (169,214,284).

In an attempt to elucidate the mechanism by which FIV would escape from VN pressure, we have used the same approach. Thus, we have identified a single amino acid change in the envelope glycoprotein of FIV that leads to the escape of the virus from neutralizing antibody.

Materials and Methods

Virus

Viral progeny of replication-competent molecular clones 19k1 (FIV19k1) and 19k32 (FIV19k32) and the uncloned isolate FIV A’dam19 were obtained as described previously (240). A 10-week-old specific-pathogen-free (SPF) cat (cat 14) was infected with FIV19k1 by intraperitoneal inoculation of 10^3 50% tissue culture infectious doses. Virus was reisolated from this cat at 2 and 32 weeks postinfection (p.i.) and designated FIV19k1R2 and FIV19k1R32, respectively.
Sera

Serum of a naturally FIV infected cat, A’dam19 was designated serum A19 (240). Serum of cat 14 was taken prior to infection (preserum) and at 22 weeks p.i. (designated serum 1422). Serum of a noninfected SPF cat was used as a control serum (SPF serum). All sera were heat inactivated for 1 h at 56 °C prior to use.

Gag- and Env-specific ELISAs

The presence of antibodies directed against the envelope protein of FIV was detected in a recombinant FIV envelope protein enzyme-linked immunosorbent assay (ELISA) as described elsewhere (218), and antibodies raised against Gag proteins were detected in a commercially available FIV p24/p17 antibody ELISA as recommended by the manufacturer (European Veterinary Laboratory, Amsterdam, The Netherlands).

RT assay

Reverse transcriptase (RT) activity was determined as previously described (240).

VN assay

Since low virus passage levels were used to avoid additional mutations by in vitro culturing, and the virus titer of the stocks propagated in feline peripheral blood mononuclear cells (PBMC) (240) was low (approximately 10^5), we decided to standardize the viral input by RT activity. Virus stocks of FIV19k1, FIV19k32, the uncloned isolate FIV A’dam19, and the reisolates FIV19k1R2 and FIV19k1R32 were tested in an RT assay. For this assay, 450 μl of diluted virus stocks containing RT activity of 5 x 10^4 cpm/ml were incubated with 50 μl of a serum sample for 60 minutes at 37 °C. concanavalin A (ConA) and interleukin-2 (IL-2)-stimulated PBMC (10^6) were added. After 60 minutes at 37 °C the cells were washed and incubated for 14 days in CM (RPMI 1640 [GIBCO], penicillin [100 IU/ml], streptomycin [100 μg/ml], L-glutamine [2 mM], β-mercaptoethanol [2 x 10^-5 M], IL-2 [100 IU/ml]) supplemented with 2% of the respective serum sample. RT activity was determined as a measure for virus production. Virus neutralization was considered positive when the RT activity did not increase over two times the background level defined as the mean RT activity of uninfected cells. In previous experiments it was observed that the RT activity of FIV-infected cultures was at least five times the background level (not shown).

Generation of escape mutants

ConA and IL-2 stimulated PBMC of an SPF cat (10^6) were infected with
Envelope protein determinants

FIV19k1. After 24 h, the cultures were divided between two identical flasks. To one of the cultures we added 2% of serum 1422, and to the other we added 2% SPF serum (control serum). The culture supernatants were harvested weekly, replaced with fresh CM supplemented with 2% concentration of the respective serum, and tested for the presence of RT activity. When RT activity over five times background values was present, the supernatant was filtered through a 220-nm-pore-size filter and stored at -135 °C. Viruses derived from these cultures were designated 19k1A and 19k1B when derived from cultures with serum 1422 or with SPF serum, respectively. Dilutions of the culture supernatant of 19k1A and 19k1B containing RT activity of 5 x 10^4 cpm/ml were incubated with 10% of serum 1422 or SPF serum for 60 minutes at 37 °C. ConA and IL-2 stimulated PBMC of an SPF cat (10^6) were added to each culture. After 60 minutes of incubation at 37 °C, the cells were washed and cultured in CM supplemented with a 2% concentration of the respective serum. The culture supernatant was tested for the presence of RT activity twice a week. When RT activity could be demonstrated, the culture supernatant was filtered through a 220-nm-pore-size filter and stored at -135 °C. The viruses were designated 19k1A,A and 19k1A,B for 19k1A propagated in the presence of serum 1422 or SPF serum, respectively, and 19k1B,A and 19k1B,B for 19k1B propagated in the presence of serum 1422 and SPF serum, respectively. 19k1A,A was further designated 19k1esc.

Sequence analysis

The sequence of the envelope gene of 19k1 has been described previously (240). The envelope gene of the escape mutant 19k1esc was amplified by polymerase chain reaction (PCR). PBMC (10^5) from the 19k1esc culture were pelleted and lysed with 100 µl K-buffer (50 mM KCl, 10 mM Tris [pH 8.4], 1.5 mM MgCl₂, 0.5% Tween-20, and 100 µg of proteinase K per ml) for 45 min at 56 °C. The reaction was stopped by incubating the mixture for 5 minutes at 95 °C. Two oligonucleotide primers were designed (primer 1, 5'-GGCGAATTCATGGCAGAAGGATTTGTAGCC-3' and primer 2, 5'-TATGCATGCTCATCCTCCCTTTCAGACATGCC-3') which contained EcoRI and SphI restriction enzyme cleavage sites (underlined), respectively, to facilitate subsequent cloning into pUC19. PCRs were carried out in a separate laboratory (to avoid contamination) in a volume of 100 µl containing 10 µl of the cell lysate, 50 mM KCl, 10 mM Tris [pH 8.4], 1.5 mM MgCl₂, 0.02% gelatin, 250 µM deoxynucleotide triphosphates, 1 µM each primer, and 2.5 U of Taq polymerase. Samples were layered over 100 µl of mineral oil to avoid evaporation and the subjected to 35 amplification cycles consisting of a denaturing
step (94 °C, 1 min.), a primer-annealing step (60 °C, 1 min.), and a primer-
extension step (72 °C, 2 min.). Negative controls (noninfected PBMC from an SPF
cat) were amplified in parallel. Amplification products were analyzed by agarose
gel electrophoresis (0.8%) for the presence of the appropriately sized DNA
fragments. Excess primers in the PCR reaction were eliminated by using Centricon-
30 microconcentrator (Amicon). DNA was digested with EcoRI and SphI and
cloned into pUC19. The subclone was used for the dideoxy nucleotide chain
termination reaction (229) of the envelope gene. The nucleotide and protein
alignments were done with Lasergene software (DNAsstar inc. London, United
Kingdom). The observed nucleotide variation of 19k1esc was confirmed by using
an independent PCR amplification to exclude Taq errors.

Generation of chimeric clones

The construction of chimeric clones is schematically represented in Fig. 1.
Restriction mapping identified a common and unique internal SphI site in both FIV
genomes. To generate recombinant viruses, the lambda clones 19k1 and 19k32 were
digested with SalI and SphI. The SalI and SphI fragments generated after digestion
were cloned into plasmid vector pUC19 from which the KpnI site has been deleted.
The plasmids containing the 5’ long terminal repeat, gag, and 5’ part of pol were
designated 5’ subclones. Plasmids containing the 3’ part of pol, env, and 3’ long
terminal repeat were designated 3’ subclones and were used to exchange internal
1,662-bp KpnI fragments between the clones 19k1 and 19k32.

The 1,662-bp KpnI fragment of clone 19k1 was also cloned into pUC19 and was
designated the 3’KK subclone of 19k1. A 144-bp NsiI - MstII fragment of the
19k1esc envelope (see above) was exchanged, as was a 144-bp NsiI - MstII
fragment of clone 19k1 in which one nucleotide was substituted by site-directed
mutagenesis at either position 1667 or position 1678. Site-directed mutagenesis was
performed by PCR on the 3’ subclone of 19k1. To that end, three oligonucleotides
were designed. Primer 1 (5’-CCTTATTATGCATTTCATATATGACAAAAGCTG-
3’) contained an NsiI site (underlined); primer mut1 (5’-GCCTTTTTCCTCAGGACATTCATTTATTGTGTGAGTATTG-3’)
contained an MstII site (underlined) and a T-to-G substitution at position 1667 (italic); primer
mut2 (5’-GCCTTTTTCCTCAGGACATTCATTTATTGTGTGAGTATTG-3’)
contained an MstII site (underlined) and a T-to-G substitution at position 1678 (italic). Two PCRs
were carried out, using in both reactions primer 1 to prime from the 5’ end. In one
reaction primer mut1 was used while in the other reaction primer mut2 was used to
prime from the 3’ end. The conditions for the PCR are described above. PCR
fragments were digested with NsiI and MstII and cloned into the 3’ KK subclone of
Envelope protein determinants

Figure 1. Schematic representation of the construction of chimeric FIV clones (see text for details). The uppermost bars represent the major open reading frames gag, pol and env. Restriction enzyme cleaving sites are indicated as: K:KpnI; M:MstII; N:NsiI; S:SaiI; Sp:SphI. Individual point mutations are indicated with "v".

19k1. The mutagenesis was confirmed by sequence analysis.

The 1,662-bp KpnI fragments from the 3' KK subclones in which 144 bp NsiI and MstII fragments were exchanged were cloned into the 3' subclone of 19k1; 2.5 μg amounts of each of the 3' subclones and the 5' subclones were mixed, digested with SphI, and ligated. After digestion with SaiI the constructs were transfected into Crandell feline kidney (CrFK) cells as previously described (240). Virus production was rescued by cocultivation of transfected cells with ConA and IL-2 stimulated PBMC for 3 days. Cultures were monitored for syncytium formation of PBMC and RT activity in the culture supernatant. When RT activity could be measured, culture supernatant was filtered through an 220-nm-pore-size filter and stored in aliquots at -135 °C.
Chapter 3

Synthesis and reaction of peptides with sera

Twelve-mer peptides with an overlapping sequence of 11 amino acids were synthesized and analyzed for reactivity with sera as previously described (81). The amino acid sequences of the peptides were based on the amino acid sequences of the envelope proteins of clones 19k1 and 19k1esc between amino acid position 549 and 583. Three 12-mer control peptides with an overlapping sequence of eight amino acids were synthesized according to the epitope between position 596 and 616 as described by Avrameas et al. (13).

Results

Infection of cats

Upon experimental infection with viral progeny of clone 19k1, cat 14 seroconverted within 4 weeks. The Gag- and Env-specific serum antibodies measured by ELISA reached plateau levels within 8 weeks after infection (Table I). Virus was reisolated 2 and 32 weeks p.i. (Table I). The serum collected 22 weeks p.i. (serum 1422) and the two virus isolates (FIV19k1R2 and FIV19k1R32, respectively) from this cat were used in the experiments described below.

Table 1. Anti FIV serum antibody response of cat #14 determined by gag- and env specific ELISA and virus isolation after infection with FIV19k1 of cat #14.

<table>
<thead>
<tr>
<th>Wk p.i.</th>
<th>antibody response</th>
<th>virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD&lt;sub&gt;450&lt;/sub&gt;</td>
<td>OD&lt;sub&gt;450&lt;/sub&gt; reciprocal titer</td>
</tr>
<tr>
<td>0</td>
<td>&lt;50</td>
<td>&lt;40</td>
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<tr>
<td>2</td>
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<td>4</td>
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<td>6</td>
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<td>8</td>
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<tr>
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<td>&gt;5120</td>
</tr>
<tr>
<td>32</td>
<td>1,036</td>
<td>&gt;5120</td>
</tr>
</tbody>
</table>

<sup>a</sup> OD<sub>450</sub> optical density at 450 nm (x 10<sup>2</sup>); <sup>b</sup> ND: not determined.
**Envelope protein determinants**

**VN activity**

Serum from cat 14 (serum 1422) was tested for VN activity against FIV19k1, FIV19k32 and the uncloned isolates FIV A’dam19, FIV19k1R2, and FIV19k1R32. SPF serum was used as a control. The results are listed in Table 2. Virus replication as determined by RT activity was demonstrated in all cultures in the presence of SPF serum. Replication of virus was detected in the presence of serum 1422 in the cultures of FIV19k32 and the uncloned isolate FIV A’dam19. However, no virus replication was detected in the culture infected with FIV19k1 and the reisolates FIV19k1R2 and FIV19k1R32 cultured in the presence of serum 1422, indicating complete neutralization. Serum 1422 which was absorbed with protein-A Sepharose beads to remove antibodies (particularly immunoglobulin G) failed to neutralize molecular clone 19k1, which indicates that serum immunoglobulin is responsible for the observed neutralizing activity (not shown).

<table>
<thead>
<tr>
<th>Virus</th>
<th>RT activity after neutralization with:</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>serum 1422</td>
</tr>
<tr>
<td>FIV A’dam19</td>
<td>24,219</td>
<td>61,480</td>
</tr>
<tr>
<td>FIV19k1</td>
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<td>FIV19k32</td>
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</tr>
<tr>
<td>FIV19k1R2</td>
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</tr>
<tr>
<td>FIV19k1R32</td>
<td>103</td>
<td>18,145</td>
</tr>
</tbody>
</table>

**Generation of escape mutants**

FIV19k1-infected PBMC from an SPF cat were cultured in the presence of serum 1422 (culture A) or SPF serum (culture B). Within 17 days, RT activity could be measured in the supernatant of culture B. After 44 days of culture, RT activity was detected in culture A (Fig. 2A). RT activity increased during time of culture. No RT activity could be demonstrated in the noninfected cultures which were cultured in the presence of serum 1422 or SPF serum for 52 days of culture (data not shown).

The supernatants of cultures A and B were collected, filtered 52 days p.i., and designated 19k1A and 19k1B, respectively. These culture supernatants were
incubated with serum 1422 or SPF serum. ConA- and IL-2 stimulated PBMC from an SPF cat were added to the virus - serum mixture and cultured further in the presence of the respective serum, serum 1422 or SPF serum. The cultures were designated 19k1A,A and 19k1A,B for the cultures of 19k1A cultured in the presence of serum 1422 or SPF serum, respectively (Fig. 2B) and 19k1B,A and 19k1B,B for the cultures of 19k1B cultured in the presence of serum 1422 or SPF

Figure 2. Kinetics of virus replication expressed in RT activity of FIV19k1 (A), FIV19k1A (B), and FIV19k1B (C) in the presence of serum 1422 (dashed line) or SPF serum (solid line).
Envelope protein determinants

serum, respectively (Fig 2C). RT activity was demonstrated after 17 days of infection in both cultures of 19k1A. The kinetics of virus replication of 19k1A,A were similar to those of the control 19k1A,B (Fig. 2B) and 19k1B,B (Fig 2C). As expected no virus replication was observed in the culture 19k1B,A (Fig. 2C). These results show that 19k1A,A is resistant to virus neutralization by serum 1422.

Sequence analysis

Cells of the culture 19k1A,A further designated 19k1esc were lysed and the envelope gene of the FIV genome, integrated in the cellular DNA, was amplified by PCR. The PCR product was digested with EcoRI and Sphi and cloned into pUC19. The envelope gene of 19k1esc was sequenced and compared with the envelope sequence of the parental clone 19k1 and clone 19k32 (Fig. 3). At position 468 in hypervariable region 2 (HV-2), we observed a silent transition of T to C (not shown). Two A-to-C transversions were observed in HV-5. One at position 1667 causes an amino acid substitution of asparagine to threonine (amino acid position 556), whereas the other at position 1678 causes an amino acid substitution of lysine to glutamine (amino acid position 560). Comparison of the nucleotide sequence of the envelope gene of clone 19k1 with 19k32, which exhibited the same phenotype as 19k1esc in a VN assay using serum 1422, showed the presence of a T-to-G transversion at position 1665 and a transition of C to T at position 1669, resulting in a substitution of asparagine with lysine (amino acid position 555) and histidine with tyrosine (amino acid position 557), respectively (Fig. 3).

<table>
<thead>
<tr>
<th>position</th>
<th>540</th>
<th>550</th>
<th>560</th>
</tr>
</thead>
<tbody>
<tr>
<td>19k1esc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19k32</td>
<td></td>
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<td></td>
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</table>

Figure 3. Comparison of the amino acid sequences of HV-5 of the envelope genes of the molecular clones 19k1, 19k32 and 19k1esc.

Virus neutralization of chimeric clones

Viral progeny of the parental clone 19k1 and of those in which the envelope gene had been exchanged partially or an amino acid had been substituted by site
Chapter 3

directed mutagenesis and the escape mutant 19k1esc were incubated with serum 1422 or SPF serum prior to infection of ConA and IL-2 stimulated thymocytes. The results of this experiment are similar to those of a number of previous experiments in which the chimeric clones were tested separately for neutralization by serum 1422. RT activity was measured 9 and 12 days p.i. RT activity could be measured in all the control cultures 9 days p.i. and continued to increase through 12 days p.i. (Fig.4). As expected, FIV19k1 was neutralized by serum 1422 and not by the SPF serum (Fig. 4A). As before, FIV19k32 was not neutralized by serum 1422 (Fig. 4B). To investigate whether the differences in virus neutralization between the two molecular clones was caused by the differences in the surface protein, we exchanged the 1,662-bp KpnI fragment. The chimeric clones were designated 19k1KK32 (19k1 containing the KpnI fragment of 19k32) and 19k32KK1 (19k32 containing the KpnI fragment 19k1). Chimeric clone 19k1KK32 was not neutralized, whereas chimeric clone 19k32KK1 was neutralized with serum 1422 (not shown), indicating that an epitope within the 1,662-bp KpnI fragment of the envelope gene is involved in neutralization.

The escape mutant of FIV19k1, grown in the presence of serum 1422 was not neutralized by this serum (Fig. 4C). Comparison of the amino acid sequence of the envelope protein of 19k1 and the sequence of the escape mutant showed two amino acid substitutions within HV-5. To study whether these substitutions underlie the escape of FIV19k1 from serum 1422, we exchanged the 144-bp NsiI - MstII fragment, containing the whole HV-5, of clone 19k1 with that of the escape mutant. This chimaera, designated 19k1NMesc was not neutralized by serum 1422 (Fig. 4D), indicating that one or both amino acid substitutions are involved in the escape mechanism. By site-directed mutagenesis, the two amino acids were substituted separately. These point-mutated clones were designated 19k1mut1 (position 556, Asn to Thr) and 19k1mut2 (position 560, Lys to Gin). FIV19k1mut1 was neutralized by serum 1422 (Fig. 4E), whereas FIV19k1mut2 could not be neutralized by serum 1422 (Fig. 4F), indicating that the point mutation at amino acid position 560 resulting in an amino acid substitution from lysine to glutamine is responsible for escape of FIV19k1 from serum 1422.

To investigate whether the differences in HV-5 between clones 19k1 and 19k32
Envelope protein determinants

Figure 4. Neutralization of viral progeny of parental and chimeric FIV clones and 19k1esc with serum 1422 (dashed line) or SPF serum (solid line) as determined by measuring RT activity. (A) FIV19k1; (B) FIV19k32; (C) FIV19k1esc; (D) FIV19kINMesc; (E) FIV19k1mut1; (F) FIV19k1mut2.

also underlie differences in neutralization with serum 1422, we reciprocally exchanged the 144 bp NsiI - Mst2 fragment between the two clones. This exchange, however, did not result in a change in virus neutralization (not shown), indicating
that yet another epitope is involved.

**Pepscan analysis**

Twelve-mer peptides representing the amino acid sequence of the clones 19k1 and the escape mutant 19k1esc between amino acid positions 549 and 583 were analyzed for reactivity with serum 1422, serum from a naturally FIV infected cat, A’dam19 (serum A19), and SPF serum. As a control for the peptide scanning (pepscan) procedure, three 12-mer peptides spanning, with an overlap of eight amino acids, a previously described linear epitope on the envelope protein of FIV (from amino acid position 596 to 616) were scanned with serum 1422, serum A19, and SPF serum. No reactivity in these sera was detected with the HV-5 peptides, whereas serum 1422 and serum A19 did react in the control pepscan (not shown) suggesting that no linear B-cell epitope in the C-terminal half of HV-5 is recognized by serum 1422 and serum A19.

**Discussion**

In this report we have demonstrated that a single amino acid substitution in the HV-5 region of the surface glycoprotein of FIV (amino acid position 560) allows a molecular clone of FIV to escape neutralization by the serum of a cat, infected with this clone. Pepscan analysis of the region around amino acid 560 with and without the substitution showed no reactivity of the peptides with sera from cats infected with the molecular clone or with an uncloned isolate of FIV from the same origin. This finding suggests that the B cell epitope involved in the escape is either dependent on its three dimensional configuration or is located outside the region where the substitution took place. In the latter case, the amino acid substitution should have altered an epitope at a distant site. Similar studies with escape mutants of molecularly cloned HIV-1 have shown that an amino acid substitution in the PND may directly result in the escape from neutralization by a PND - specific monoclonal antibody (169). Others have shown that an amino acid substitution in the transmembrane glycoprotein of HIV-1 induced resistance to neutralization by
sera from seropositive individuals (214). The basis for this resistance was shown to be most likely a conformational change altering a neutralization inducing determinant at a distant site (284). To elucidate the mechanism involved in the escape from neutralization observed in the FIV system, further studies identifying antigenic sites that induce virus neutralization will be necessary.

Our data, which showed that an FIV escape mutant could readily be generated in the presence of VN serum antibody, suggest the predominance of type specific VN antibodies in serum 1422. The finding that one amino acid substitution allowed FIV19k1 to escape from neutralization by the cat serum collected 22 weeks p.i. indicates that this substitution affects a dominant VN antibody-inducing determinant against which the majority of VN antibodies in this serum are directed during the first months of infection. Another explanation would be that this substitution influences more than one VN antibody-inducing epitope. The observation by Tozzini et al. (271) and Fevereiro et al. (69) that within 4 weeks after FIV infection, serum antibodies with broad VN activity can be detected suggests that as in HIV-1 infection of humans, broadly reactive VN antibodies are formed. It should, however, be stressed that the VN assays that they used were carried out with FIV strains adapted to replication in a continuous fibroblastoid feline kidney cell (CrFK) line, whereas our VN assays were carried out in primary cat lymphocytes or thymocytes. Lymphoid cells, which are the natural target cells for FIV replication in vivo, can readily be infected with all strains of FIV, whereas only few FIV strains could be adapted to replication in CrFK cells. The genetic basis for this adaptation is currently unknown, but since upon transfection with proviral DNA, CrFK cells produce FIV that cannot reinfect CrFK cells, virus entry is probably the limiting step. Therefore it may be speculated that the broad neutralization observed in the assays based on CrFK cell adapted FIV strains specifically interferes with the process underlying this limiting step.

Our future studies to elucidate the structural basis of FIV neutralization will also be based on the observations made with FIV19k32. This virus, like FIV19k1esc was not neutralized by serum 1422. Exchange of the NsiI - MstII fragment of 19k1, for the homologous fragment of 19k32, which contains the HV-5 region, could not confer resistance to 19k1. Since exchanging the 1,662-bp KpnI fragment between
19k1 and 19k32 resulted in the transfer of the neutralization resistant phenotype and the envelope proteins of 19k1 and 19k32 differ in only six amino acids, two of which are located within the HV-5 region (Fig. 3), genetic exchange experiments will reveal the exact location of additional regions involved in escape from virus neutralization.

As in other lentivirus infections, VN antibodies and cytotoxic T cells may be expected to play a crucial role in the pathogenesis of and the protective immunity against FIV infections. Therefore the detailed analysis of antigenic sites of FIV involved in the induction of VN antibodies and the mechanism leading to VN-resistant FIV variants will lead to a better understanding of lentivirus pathogenesis and help in the design of effective lentivirus vaccines.

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Neutralization of Feline Immunodeficiency Virus by Polyclonal Feline Antibody: Simultaneous Involvement of Hypervariable Regions 4 and 5 of the Surface Glycoprotein

Kees H.J. Siebelink, Willem Huisman, Jos A. Karlas, Guus F. Rimmelzwaan, Marnix L. Bosch, and Albert D.M.E. Osterhaus

Abstract

Sites involved in antibody mediated neutralization of feline immunodeficiency virus were mapped by reciprocal exchange of envelope fragments or amino acids between molecular clones of feline immunodeficiency virus with different susceptibilities to neutralization by a polyclonal cat serum. Combinations of mutations within HV-4 or within HV-4 and HV-5 changed the susceptibility of the viruses to neutralizing antibody.

Feline immunodeficiency virus (FIV) is a lentivirus that causes a severe impairment of immune function in experimentally and naturally infected cats, which eventually results in the development of a syndrome quite similar to AIDS in humans (2,16,53,104,115,143,199,236,241,248,289). For both human immunodeficiency virus type 1 and FIV it has been shown that single amino acid substitution mutations in the envelope glycoprotein, within or outside the presently identified VN epitopes, may confer resistance to virus neutralizing (VN) monoclonal or polyclonal antibody (126,169,242,243,284). It has also been postulated that glycosylation may play an important role in the proper expression or masking of VN epitopes (6,26,48,89,93).

We have recently described two molecular clones of FIV (FIV19k1 and FIV19k32), simultaneously derived from a naturally infected cat (240). These clones differed in their susceptibilities to virus neutralization in feline thymocytes by serum S1422, obtained from a specified-pathogen-free cat, 22 weeks after infection with FIV19k1 (242). The surface glycoproteins (SU) of the two molecular clones were shown to differ in only five amino acids (240). We have demonstrated that a single substitution mutation at amino acid position 560 (HV-5) or at position 483 of FIV19k1 conferred resistance to virus neutralization by S1422 (242,243). In the present study we have further evaluated the involvement of different sites of the SU protein in antibody mediated neutralization by reciprocal exchange of FIV19k1 and FIV19k32 envelope gene fragments and by site directed mutagenesis. This included a highly conserved potential N-linked glycosylation site at amino acid position 481 (245).

Combinations of point mutations were made in the SU protein of FIV19k32, on basis of the FIV19k1 sequence, in order to obtain a virus neutralization sensitive
mutant of FIV19k32. To this end site-directed mutagenesis and gene fragment exchange were carried out as previously described (242,243). Replication competence of the molecular and chimeric clones was confirmed by showing that the production of FIV antigen in independent duplicate thymocyte cultures was in the same order of magnitude as FIV antigen production of cultures infected with FIV19k1 or FIV19k32 (less than 10^{0.5} times difference), under previously described conditions (242,243). Two virus neutralization sensitive chimeric viruses, FIV19k32mutA (Asp-454 to Asn and Ser-483 to Leu) and FIV19k32mutB (Ser-483 to Leu and HV-5 region of 19k1) were thus constructed. The VN indices of SI422 for the respective viruses were measured as previously described (242,243) and were 14 and 25 on day 8 and 5 and 9 on day 12 (Fig. 1A). FIV19k1 and FIV19k32 and the chimeric viruses FIV19k1mutA, FIV19k1mutB, FIV19k32mutC, FIV19k32mutD and FIV19k32mutE, which had been generated previously and contained only one or two point mutations compared with their parent viruses, were also tested in the same experiment. Essentially the same results were obtained as in previous studies (242,243) (Fig. 1A). These data show that a point mutation reciprocal to the Leu to Ser mutation at amino acid position 483, which has been shown to render FIV19k1 insensitive to neutralization by SI422 (243), renders FIV19k32 sensitive to this neutralization only if at least an additional mutation at amino acid position 454 is created or additional exchange of HV-5 is achieved (Fig. 1A).

The Leu to Ser substitution at amino acid position 483 of FIV19k1 introduces a potential N-linked glycosylation site at position 481. To investigate the role of this potential N-linked glycosylation in the mechanism of neutralization and escape, 12 chimeric clones were constructed by site-directed mutagenesis (Fig. 1B). To test whether the Ser at amino acid position 483 itself was important, we substituted in the SU protein of FIV19k1, FIV19k32, FIV19k32mutA and FIV19k32mutB the Leu by a Thr at this position. This resulted in chimeric clones FIV19k1mutD, FIV19k32mutF, FIV19k32mutA1 and FIV19k32mutB1, respectively with a potential N-linked glycosylation site in the absence of a Ser at this position. Replication of FIV19k1mutD in presence of SI422 proved to be inhibited (Fig. 1B). Thus independent from the potential N-linked glycosylation site, the actual amino acid present at amino acid position 483 does influence the SI422 virus neutralization phenotype of the virus. The VN phenotypes of chimeric clones FIV19k32mutF, FIV19k32mutA1 and FIV19k32mutB1 were similar to that of the parent virus FIV19k32: None of these chimeric viruses were neutralized by SI422 (Fig. 1B).

To disrupt the potential N-linked glycosylation site at position 481 in the SU protein of FIV19k1mutB, FIV19k32, FIV19k32mutC and FIV19k32mutE the Asn at amino acid position 481 was substituted by a Gln, another uncharged polar amino acid. This substitution significantly reduced virus production in thymocyte cultures infected with these viruses, and the resulting viruses were resistant to neutralization by SI422 (Fig. 1B).
Figure 1. Schematic representation of parts of the HV-4 and HV-5 regions of FIV19k1 and FIV19k32 and chimeric viruses constructed by exchange of single or multiple amino acids in the HV-4 and HV-5 regions of FIV19k1 and FIV19k32 (A) and by altering the potential N-linked glycosylation site at amino acid position 481 (B). SI422 VN indices measured on days 8 and 12 against the respective viruses are also shown (*, data from reference 20). The envelope glycoprotein is diagrammed at the top. The hypervariable regions are numbered 1 through 5 in the top box. Restriction enzyme cleaving sites: K, KpnI; M, MstI; N, NsiI. Open and shaded bars, sequence of FIV19k1 and FIV19k32, respectively. The amino acid sequence of clone FIV19k1 is shown. The individual mutated (and relevant nonmutated) amino acids are indicated, with shading and lack of shading indicating the virus of origin (see above). Amino acids which alter the potential N-linked glycosylation site and which are not present in FIV19k1 or FIV19k32 are highlighted in black.
Envelope protein determinants

acid. This resulted in chimeric viruses FIV19k1\textsuperscript{mutD}, FIV19k32\textsuperscript{mutQ}, FIV19k32\textsuperscript{mutC1} and FIV19k32\textsuperscript{mutE1}, respectively. None of these viruses were neutralized by S1422 (Fig. 1B).

Finally, to control for a potential virus neutralization site disruption by the presence of a Gln at amino acid position 481, the Asn at amino acid position 481 of clones FIV19k1, FIV19k32\textsuperscript{mutD}, FIV19k32\textsuperscript{mutA} and FIV19k32\textsuperscript{mutB} was also substituted by a Gln, resulting in chimeric clones FIV19k1\textsuperscript{mutE}, FIV19k32\textsuperscript{mutD1}, FIV19k32\textsuperscript{mutA2} and FIV19k32\textsuperscript{mutB2}, respectively. With the exception of FIV19k32\textsuperscript{mutA2}, the introduction of Gln at this position did not change the S1422 virus neutralization phenotype of these viruses significantly. The replication of FIV19k32\textsuperscript{mutA2} proved to be slightly inhibited. The neutralization of the parent virus of FIV19k32\textsuperscript{mutB2} was not significantly different from neutralization of the mutated virus (Fig. 1B).

In the experiments presented in this article we have used a thymocyte virus neutralization assay, since viral progeny of molecular clones 19k1 and 19k32 replicates only in feline T cells, a primary target for FIV infection. It has been shown that sera from FIV infected cats that neutralize FIV infection of CrFK cells with CrFK adapted FIV strains fail to neutralize FIV infection of thymocytes, peripheral blood mononuclear cells, or a T-lymphoid cell line (14,242,272). The FIV neutralizing activity demonstrated in the CrFK virus neutralization assay proved to be mediated predominantly by antibodies directed to an immunodominant epitope in HV-3 (50,148). This suggests that HV-3 specific antibodies largely fail to neutralize FIV in the thymocyte virus neutralization assay, and that the HV-3 domain, if at all, is only indirectly involved in the mechanisms of virus neutralization in feline thymocytes.

Be it as it may, in the present article we have shown that both the HV-4 and HV-5 domains of two closely related FIV clones are directly involved in FIV neutralization in feline thymocytes. Using bacterial expressed envelope fragments of FIV it was shown that the HV-4 region contains a highly type specific antigenic site (50,198). However, in pepscan analysis of both regions using 12-mers we could not obtain evidence for true linear epitopes in these regions (242,243) (unpublished data), indicating that the epitopes involved are to a certain extent conformation dependent. It is interesting to note that the location of the regions involved in FIV neutralization identified here is reminiscent of the CD4 binding site of human immunodeficiency virus type 1, which is a discontinuous site with contributions mostly from the carboxy terminal half of the SU protein. Antibodies to the CD4 binding site have VN activity, just like the antibodies present in serum S1422. On the basis of these similarities it may be hypothesized that the HV-4 and HV-5 regions of the SU protein contribute to the definition of the binding site for the
cellular receptor of FIV.

Together with previously reported results (242,243) the data presented show that mutations in the HV-4 and HV-5 regions influence the susceptibility of FIV to antibody mediated neutralization.

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Chapter 4

Vaccine Development
Removal of the Cleavage Site of Recombinant Feline Immunodeficiency Virus Envelope Protein Facilitates Incorporation of the Surface Glycoprotein in Immune-Stimulating Complexes

Guus F. Rimmelzwaan, Kees H.J. Siebelink, Robin C. Huisman, Bernard Moss, Michael J. Francis, and Albert D.M.E. Osterhaus

Abstract

Recombinant vaccinia viruses were constructed that expressed the complete env gene of feline immunodeficiency virus with or without the nucleotide sequence encoding the cleavage site between the surface (SU) protein and the transmembrane (TM) protein. Removal of the cleavage site resulted in the expression of a 150 K protein that is processed to a 130 K protein and was not cleaved into the SU and the TM proteins. Removal of the cleavage site facilitated incorporation of the SU protein in immune stimulating complexes (iscoms). Antibody responses to both an SU and a TM peptide representing two immunodominant B cell epitopes were measured. These were higher in cats immunized with iscoms prepared from the cleavage site deleted envelope protein than in cats immunized with iscoms prepared from the native envelope protein or immunized with the envelope protein and the adjuvant Quil A.

Feline immunodeficiency virus (FIV) is a lentivirus that was first isolated from domestic cats which displayed clinical signs similar to those found in humans with AIDS (199). Like human and simian lentiviruses, FIV is T lymphotropic, causes a loss of CD4+ T cells, can infect macrophages and astrocytes, persists in infected cats and causes immunodeficiency in its natural host (10,30,53,104,241,269). Because of the similarities found between FIV infection in cats and human immunodeficiency virus (HIV) infection in humans, feline AIDS has been considered to be a useful small-animal model for the evaluation of strategies for the development of antiviral compounds and experimental vaccines to combat human AIDS.

The env gene encoded surface (SU) and transmembrane (TM) glycoproteins of lentiviruses play a crucial role in the process of virus-cell attachment and fusion and serve as targets for neutralizing antibodies and cytotoxic T cells which are major components of antiviral immunity. Consequently, for the development of vaccines against FIV infections in cats, the incorporation of FIV glycoproteins presented in a form that stimulates both cell- and antibody-mediated immunity should be considered. It has been shown that immune-stimulating complexes (iscoms) induce both virus-neutralizing antibodies (51,177,194) and MHC class I-restricted CD8+ cytotoxic T lymphocytes (CTL) against retrovirus glycoproteins (256). Furthermore, we and others have recently documented that the iscom presentation form allows
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the endogenous processing which is necessary for both MHC class I- and class II-
restricted recognition by CD8+ and CD4+ T cells, respectively (98,180,274).
Therefore, we have chosen the iscom structure as a basis for our strategy for the
development of an FIV vaccine.

The incorporation of proteins into iscom is largely facilitated by the presence of
regions which allow hydrophobic interaction with the iscom matrix (39). Since only
the TM protein of FIV env is highly hydrophobic and the non-covalently bound TM
and SU proteins readily dissociate, our strategy was to incorporate an FIV env
protein from which the cleavage site between the SU and TM proteins had been
deleted. We generated a recombinant vaccinia virus (rVV) that expresses an FIV
env protein from which this cleavage site has been deleted in order to facilitate
incorporation of the SU protein in the iscom matrix.

The FIV env gene (2.6 Kb) encoding the env precursor protein was amplified by
PCR. Bone marrow-derived DNA, obtained from an FIV-infected cat was used as a
source of FIV proviral DNA (240). The oligonucleotides for PCR amplification
were synthesized on an Applied Biosystems DNA synthesizer. They were based on
the nucleotide sequence of the Petaluma strain of FIV (259): 5’GGCAGTTTGAATCTACATTATC3’ and 5’GCAACAAATAAAGAATGGCAG3’
(prime from the 5’ end), 5’CTCACAGATCATCTCC3’ and 5’GACATAACCTCTCACAAGGG3’ (prime from the 3’ end). The amplified
fragment was cloned into the HincII site of the cloning vector pBluescript II SK(+) (Stratagene). The FIV env gene was excised from the pBluescript construct
(pBS/env), with the restriction enzymes XhoI and SmaI, and cloned into the Sall
and SmaI-digested plasmid pSC65 (S. Chakrabarti and B. Moss, unpublished
results). This plasmid contained vaccinia virus thymidine kinase (TK) sequences
flanking the cloning site, a synthetic early/late vaccinia virus promotor and the
lacZ gene under control of the vaccinia 7.5 K promotor. The resulting plasmid was
designated pGR657. Plasmids were screened by restriction endonuclease analysis
and DNA hybridization using gel-purified env gene as labeled probe (ECL;
Amersham) and were grown by standard procedures. For the construction of an
rVV that expresses the env precursor protein without the cleavage site between the
SU and TM proteins, the nucleotide sequence at position 1822 to 1833, coding for
the potential cleavage site RRKR, was deleted. To this end, 3 μg of the pBS/env
was used as template DNA for amplification by PCR using primers 5’-del
(5’GGAAGTCATGGGAATATAAACCTGCAGCTATTTCATGGTATGTTGGC3’; nt
1800 to 1856 from which nt 1822 to 1833 were deleted) and 3’ BglII
(5’GGGTTAGATCTTTTGTGGTATACC3’; nt 2204 to 2227), to obtain
fragment 5’-del/3’-BglII. Using primer pair 3’-del (reverse complementary to 5’-
del) and 5'-NsiI (5'GACCTTATTGCATTTCATATGACAAAAAGCTG3'; nt 1534 to 1567), fragment 5'-NsiI/3'-del was obtained. To obtain the 5'-NsiI/3'-BglII fragment with the deletion, a PCR was performed with the 5'-NsiI and 3'-BglII primers and 500 ng of the fragments 5'-NsiI/3'-del and 5'-del/3'-BglII as template. The PCR-derived 5'-NsiI/3'-BglII fragment was purified by preparative gel electrophoresis, digested with NsiI and BglII and cloned into NsiI/BglII-digested pGR657. The resulting plasmid was designated pGR657X15. The presence of the deletion was confirmed by determination of the nucleotide sequence in this region using the dideoxynucleotide chain termination sequencing.

Subsequently rVV's were generated as previously described (155) by homologous recombination with vaccinia virus (WR strain), which was obtained originally from the ATCC. Recombinant virus plaques were visualised by their blue color as a result of the co-expression of the lacZ gene and overlay with X-gal (36). The viruses were plaque-purified three times and stocks of vGR657 and vGR657X15 were grown in Hela cells.

The FIV proteins expressed by vGR657 and vGR657X15 were analyzed by pulse-labeling in a radioimmunoprecipitation assay (RIPA) and Western blot analysis. For RIPA Hela cells were infected with vGR657, vGR657X15 or vSC65 (control rVV made with pSC65) at an m.o.i. of 30 p.f.u. per cell. At 4 h post infection the cells were incubated in methionine- and cysteine-free medium for 30 min, after which they were pulse-labeled for 30 minutes with [35S]methionine and [35S]cysteine (200 μCi/ml), followed by different chase periods (0, 2 and 24 h). FIV-specific proteins were immuneprecipitated from both culture supernatants and cell lysates with polyclonal anti-FIV antibody (serum from the naturally infected cat Adam 19; (240) and a monoclonal antibody (MAb) 6-13-12, specific for FIV TM protein (see below).

For the generation of this MAb, BALB/c mice were immunized with iscoms in which the FIV envelope proteins expressed by rVV vGR657 were incorporated. For incorporation in iscoms, lysates of vGR657-infected cells were solubilized in PBS containing 2% MEGA-10 (Boehringer Mannheim). Subsequently, glycoproteins were purified by affinity chromatography using lentil lectin-Sepharose (Pharmacia). The purified protein was mixed with the lipids cholesterol and phosphatidylethanolamine (Sigma) and Quil A (Spikoside; ISCOTEC) at a ratio of 1:1:5 (w/w) and after ultrasonication for 30 s incubated for 1 h at room temperature in the presence of 0.2% MEGA-10. The mixture was then dialyzed against PBS for 16 h at room temperature followed by dialysis against PBS for 24 h at 4°C. The resultant iscom were analyzed by electron microscopy, revealing the typical cage-like structure, and also by SDS-PAGE followed by Western blot analysis. This provided evidence for the predominant incorporation of TM protein (not shown).
Figure 1. Pulse-chase analysis of the generation of FIV proteins after rVV infection of Hela cells. After infection with rVV vSC65, vGR657 or vGR657X15, cells were labeled with [35S]methionine and [35S]cysteine. At 0, 2 and 24 hours after labeling (lanes 1, 2 and 3 respectively) FIV env proteins in cell lysates and culture medium were visualized by immune precipitation and PAGE using (a) MAb 6-13-12 and (b) serum from FIV-seropositive cat Adam 19.
BALB/c mice were immunized with these iscoms and hybridomas were generated according to standard procedures (195). For the identification of hybridomas producing antibodies reactive with FIV env proteins an FIV env-specific ELISA was used (218). MAb 6-13-12, selected for these studies, proved to be specific for the TM protein of FIV as demonstrated by its reactivity with FIV env proteins in RIPA (Fig. 1a) and immunostaining of Western blots (Figs. 2a and 3a).

Directly after pulse labelling of cells infected with vGR657 two FIV proteins with $M_r$'s of 150K and 130K were observed in the cell lysates by immunoprecipitation with the serum of cat Adam 19. After a chase period of 2 h
the 150K protein had disappeared and only the 130K protein remained. Twenty four h after labelling a smear of protein with a mean $M_\text{r}$ of 32K and a 95K protein were visualized in addition to the 130K protein. In the supernatant of the culture infected with vGR657 only a 95K protein was only observed (Fig. 1b).

These results show that in cells infected with rVV vGR657 a 150K FIV precursor envelope protein is synthesized which is then processed into a 130K protein. This protein is subsequently cleaved into a 95K protein that is released into the culture supernatant and most likely represents the SU protein and a 32K protein which most likely represents the TM protein. Thus the processing of FIV glycoproteins expressed by rVV appears to be similar to the processing of FIV glycoproteins in persistently infected Crandell feline kidney (CrFK) cells as described by Stephens et al. (254).

In the cell lysate fraction of cells infected with rVV vGR657X15 FIV proteins with $M_\text{s}$ of 150K and 130K were also present directly after pulse labelling. After a chase period of 2 h the 130K protein proved to be predominantly precipitated and after a chase period of 24 h this was the only FIV protein observed. No FIV protein was precipitated from the supernatants of the cultures infected with rVV vGR657X15 (Fig. 1b). These data suggest that in cells infected with vGR657X15, an FIV precursor envelope protein with an $M_\text{r}$ of 150K was synthesized and that this protein was processed into a 130K protein that is not cleaved like the native protein. Immunoprecipitation studies with MAb 6-13-12 using the same samples confirmed this suggestion. With this MAb, the 150K, 130K and 32K proteins, but not the 95K protein, were immunoprecipitated from lysates of cells infected with rVV vGR657 (Fig. 1a). Proteins with $M_\text{s}$ of 150K and 130K were precipitated from cells infected with rVV vGR657X15. No 32K protein was precipitated from these lysates, indicating that the 130K protein expressed by vGR657X15 is not cleaved.

When lysates of rVV infected BHK cells were tested for the presence of FIV proteins by Western blot analysis, the 130K protein could be detected in vGR657X15-infected cells but not in vSC65- or vGR657-infected cells. In vGR657-infected cells the TM protein could be detected but it was not detected in vSC65- or vGR657X15-infected cells (Fig. 2), which confirms the findings of the pulse labelling experiment.

Incorporation studies with lentil lectin purified extracts from BHK cells infected with the respective rVVVs showed that iscom prepared from purified extracts of rVV vGR657X15-infected cells (as described above) predominantly incorporated the whole uncleaved 130K protein, in addition to minor amounts of TM protein (32K protein), as shown in Western blot analysis using MAb 6-13-12 (Fig. 3a) or serum
from seropositive cat Adam 19 (Fig. 3b). We speculated that the presence of the 32K protein was the result of a putative secondary cleavage site and that this protein was found now as a result of the concentration of the hydrophobic proteins during the process of iscom formation. In contrast, iscoms prepared from similarly purified extracts of vGR657-infected cells incorporated no 130K protein, but did incorporate the TM protein and a 64K protein that was speculated to represent a dimer of the TM protein (Fig. 3). The 130K protein incorporated in the iscom matrix is of particular interest as an immunogen since it may be expected to contain the majority of the T and B cell epitopes of the native envelope proteins, although it cannot be ruled out that the conformation of this protein has changed by deletion of the cleavage site. However, it was recently shown that an rVV expressed gp160 of HIV-1 from which the cleavage site had been deleted still bound soluble CD4 (54) or membrane bound CD4 (123), indicating that at least the conformation dependent CD4-binding site had retained its conformation in this protein.

The immunogenicity of these candidate FIV vaccines was tested in cats. Three groups of six cats were immunized subcutaneously twice with 4 week interval. Each vaccine dose contained either 10 μg vGR657 iscoms, 10 μg vGR657x15 iscoms or 10 μg vGR657x15 as soluble protein mixed with 20 μg Quil A. A fourth group was immunized twice in the same way with 10 μg of control iscoms prepared with cleavage site deleted simian immunodeficiency virus (SIVmac) envelope protein, essentially in the same way as described for vGR657x15 iscoms. Plasma samples collected at day 0, 28 and 56 post-immunization were tested in serial twofold dilutions for the presence of antibodies against two synthetic peptides in an ELISA. The peptides were a kindly gift from R. van Herwijnen, European Veterinary Laboratory, Woerden, The Netherlands. The first peptide contained the linear virus neutralization site within variable region 3 comprises amino acid residues 396 to 412 of the surface protein of the FIV Petaluma strain (SU peptide) (148), and the second peptide comprised a B cell epitope between amino acid positions 824 and 848 of the transmembrane protein of the same FIV strain (TM peptide) (13). Antibody titers in plasma samples were calculated by determining the highest dilution at which the OD450 was still higher than three times the OD450 of the plasma sample from the same cat taken prior to immunization.

All six cats immunized with vGR657x15 iscoms showed plasma antibody titers ranging from 50 to 200 to both the SU and TM peptides 56 days post-immunization (Fig. 4). Five of six cats immunized with vGR657 iscoms showed plasma antibody titers ranging from 50 to 200 to the SU peptide, whereas no plasma antibodies to the TM peptide could be demonstrated. This is surprising since these iscoms contained TM protein and no detectable SU protein. One explanation for this
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Figure 4. Plasma antibody titers of individual cats immunized with vGR657 iscoms, vGR657x15 iscoms, vGR657x15 soluble protein mixed with Quil A or SIV env iscoms, against surface (SU) and transmembrane (TM) peptides, measured by ELISA at day 56 after the first immunization.

observation may be that the conformation of the epitope presented by the TM peptide was not properly exposed in vGR657 iscoms. Apparently the vGR657 iscoms still contained enough residual SU protein to induce an SU-specific antibody response. Only three of the six cats immunized with vGR657x15 soluble protein mixed with Quil A developed plasma antibodies to the SU peptide with titers ranging from 100 to 200, whereas no antibodies to the TM peptide could be detected. None of the cats immunized with the control SIV envelope iscoms developed detectable plasma antibodies to either of the peptides. Thus the cleavage
site-deleted envelope protein incorporated into the iscom matrix proved to be most efficient in inducing antibodies to both the SU and TM proteins.

The antibody responses will be analyzed further to evaluate whether antibodies to other epitopes or virus neutralizing antibodies have been induced. The evaluation of vGR657x15 iscoms as a potential candidate FIV vaccine is the subject of our present studies.

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Chapter 4.2

Enhancement of Feline Immunodeficiency Virus Infection after Immunization with Envelope Glycoprotein Subunit Vaccines


Abstract

Cats were immunized three times with different recombinant feline immunodeficiency virus (FIV) candidate vaccines. Recombinant vaccinia virus (rVV)-expressed envelope glycoprotein with (vGR657) or without (vGR657x15) the cleavage site and an FIV envelope bacterial fusion protein (β-Galactosidase-Env) were incorporated into immune-stimulating complexes or adjuvanted with Quil A. Although all immunized cats developed antibodies against the envelope protein, only the cats vaccinated with the rVV-expressed envelope glycoproteins developed antibodies which neutralized FIV infection of Crandell feline kidney cells. These antibodies failed to neutralize infection of thymocytes with a molecularly cloned homologous FIV. After the third immunization the cats were challenged with homologous FIV. Two weeks after challenge the cell-associated viral load proved to be significantly higher in the cats immunized with vGR657 and vGR657x15 than in the other cats. The cats immunized with vGR657 and vGR657x15 also developed antibodies against the Gag proteins more rapidly than the cats immunized with β-Galactosidase-Env or the control cats. This suggested that immunization with rVV-expressed glycoprotein of FIV results in enhanced infectivity of FIV. It was shown that the observed enhancement could be transferred to naive cats with plasma collected at the day of challenge.

Introduction

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that causes feline AIDS, which is similar to AIDS in humans (10,16,53,104,115,199,236,241, 248,289). The similarities between FIV and human immunodeficiency virus (HIV) on the one hand and between the pathogeneses of the syndromes they cause on the other hand have led to the use of FIV infection of cats as an animal model to evaluate the potential of preventive and therapeutic measures for HIV infection in humans. Since FIV infection is widespread among cats all over the world, the development of preventive and therapeutic measures for feline AIDS is also of major veterinary importance.

Different vaccination strategies for lentivirus infections have been evaluated with varying degrees of success. Most attempts to develop candidate vaccines against
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Lentivirus infections were without success (for a review, see reference 135). Nevertheless, in some experiments chimpanzees were successfully vaccinated against HIV type 1 (HIV-1) (20), and macaques were successfully vaccinated against HIV-2 or simian immunodeficiency virus (SIV) infections with candidate whole inactivated virus, live attenuated virus, recombinant virus, subunit virus vaccines, or combinations of these candidate vaccines (34,47,113,120,192,209,211, 235). The mechanisms by which protective immunity was established in these systems are not well understood at present, although there are indications for a major role of both virus neutralizing (VN) antibodies and cell-mediated immunity (64,144,210). However, contradictory data have also been obtained in some of these studies (12,21,110,231). In the macaque SIV model it was shown that at least part of the protective immunity induced could be attributed not to virus-specific, but rather to cell-specific antibodies (141,255). In the SIV system it was recently shown that vaccine-induced protection against infection with SIV-infected cells correlated with the presence of a certain major histocompatibility complex class I genotype of the monkeys, indicating the involvement of major histocompatibility complex class I-restricted cytotoxic T-lymphocyte responses (99).

Several approaches to develop a preventive vaccine against FIV infection have also proven to be unsuccessful (110,278). However, Yamamoto et al. reported the induction of protective immunity in cats against homologous and to a lesser extent also to heterologous FIV challenge, by vaccination with inactivated whole virus or FIV-infected cells (286,287). This protective effect could be transferred to naive cats with plasma from vaccinated animals, indicating that antibodies may be at the basis of this protective immunity (105). It was shown that serum antibodies against FIV envelope glycoproteins, with different VN domains, correlated more with protective immunity than antibodies to other viral proteins.

FIV vaccines based on recombinant envelope proteins would have clear advantages over inactivated or attenuated virus vaccines. However, so far vaccination strategies using FIV purified envelope glycoproteins or fractions of these proteins as immunogens have failed (110,149). Here we report the results of a series of vaccination experiments in cats with different envelope proteins of FIV, expressed by recombinant vaccinia viruses (rVV) or as a bacterial fusion protein and presented in the context of different adjuvant systems. The most striking finding of these studies is that vaccines containing intact envelope glycoprotein induced enhancement of infectivity rather than protective immunity against homologous FIV infection. The observed enhancement could be transferred to naive cats with the plasma of the vaccinated cats.
Materials and Methods

Cells and challenge virus

Peripheral blood mononuclear cells (PBMC) and thymocytes were derived from an 8-weeks-old specific-pathogen-free (SPF) cat (240). These cells were stimulated with concanavalin A (5 µg/ml) in culture medium (RPMI 1640 [GIBCO, Gaithersburg, MD.], penicillin [100 IU/ml], streptomycin [100 µg/ml], L-glutamine [2 mM], 2-mercaptoethanol [2 x 10^{-5} M], interleukin-2 [100 IU/ml]) and 10% fetal calf serum. After 3 days the cells were washed and cultured further in culture medium. An FIV-susceptible clone of Crandell feline kidney (CrFK) cells, named CrFK IDIO, was kindly provided by N. Pedersen (289). The FIV AM19 strain was isolated from PBMC of a cat naturally infected with FIV (240). Concanavalin A- and interleukin-2 stimulated cells were infected with FIV AM19. When FIV antigen was detected in culture supernatant by enzyme-linked immunosorbent assay (ELISA) (239), it was filtered through a 220 nm-pore-size filter, aliquoted, and stored at -135°C. This FIV stock was titrated in vivo. Groups of four SPF cats were inoculated intramuscularly (i.m.) with 0.5 ml of 1:100, 1:400, 1:1,600, 1:6,400, or 1:25,600 dilutions of the FIV stock. All cats receiving 1:100 and 1:400 dilutions became infected by 4-8 weeks postinfection as shown by seroconversion and virus isolation. Three out of the four cats inoculated with 1:1,600-diluted FIV stock and one out of four cats inoculated with 1:6,400-diluted stock became virus isolation positive and seropositive. One cat 50% cat infectious dose (CID_{50}) was therefore estimated to be 0.5 ml of a 1:3,200 dilution of the FIV stock.

FIV AM6c was isolated from PBMC of a cat naturally infected with FIV and adapted to replicate in CrFK ID10 cells as previously described (244). env gene sequence homology of FIV AM6c and FIV 19k1 is 94.8%. CrFK ID10 cells were infected with FIV AM6c, and after 6 days the culture medium was refreshed. After another 4 days of culture, when FIV antigen was detected in the culture supernatant, it was filtered through a 220-nm-pore-size filter and stored in aliquots at -135°C. This FIV stock was titrated in CrFK ID10 cells and the highest dilution (20 TCID_{50}s) which consistently resulted in detectable antigen production within 8 days was used in the VN assay (see below).

Preparation of candidate FIV vaccines

The envelope glycoproteins of FIV AM19, were expressed by an rVV in BHK cells either in their native form (vGR657) or after deletion of the cleavage site between the surface (SU) and the transmembrane (TM) proteins (vGR657x15) to facilitate incorporation into immune-stimulating complex (iscoms) (219). After
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Lectin purification these proteins were incorporated into iscoms, which resulted in two iscom preparations - vGR657 and vGR657x15 iscoms - as previously described (219). An 1,870-bp fragment of the envelope gene was excised from pBluescript, containing the whole envelope gene, by using the restriction enzymes BamHI and BglII (nucleotide position 350 and 2220, respectively) and subcloned into BamHI-digested pEX vector (Stratagene, La Jolla, Calif.). This vector allows inducible expression of proteins as β-galactosidase (β-Gal) fusion protein. This protein was partially purified as inclusion bodies, solubilized and mixed with Quil A as an adjuvant.

SIV envelope glycoprotein iscoms were prepared by E. Hulskotte using a method similar to that used for the cleavage site-deleted FIV envelope glycoprotein iscoms (114).

Vaccination and challenge infection of cats

Six groups of six SPF cats each were vaccinated three times subcutaneously according to the following schedule: group 1, vGR657 iscoms; group 2, vGR657x15 iscoms; group 3, vGR657x15 plus Quil A; group 4, β-Gal-FIV Env plus Quil A; group 5, SIV Env iscoms; and group 6, phosphate buffered saline. The cats were vaccinated with 10 µg of protein at week 0, 4 and 10. Two weeks later the cats were challenged by the intramuscular route with 20 CID₅₀s of FIV AM19. PBMC and plasma samples were collected every 2 weeks postchallenge (p.c.) during an 8-week period.

Serological assays

Antibodies against the Gag proteins p24 and p17 were detected with a commercially available test kit, using recombinant p24 and p17 proteins (catalog number F1002-AB01; European Veterinary Laboratory B.V., Woerden, The Netherlands). Antibody titers were expressed as the optical density at 450 nm (OD₄₅₀) value of the serum multiplied by the dilution divided by three times the OD₄₅₀ value of the negative control serum.

Antibodies against the envelope protein were detected by ELISA using synthetic peptides and bacterial fusion proteins, representing different regions of the envelope protein as shown in Fig. 1. The synthetic peptides were purchased from European Veterinary Laboratory B.V. (catalog numbers: SU peptide, EVS-000-PE-003; TM peptide, EVS-000-PE-004). The first synthetic peptide contains the immunodominant VN epitope within variable region 3 spanning amino acid residues 396 to 412 of the surface protein of the Petaluma strain (SU peptide) (148). The second peptide contains a B-cell epitope between amino acid positions 695 and 706.
of the transmembrane protein of the same FIV strain (TM peptide). Antibody titers were calculated as described for the Gag ELISA.

An ELISA based on the detection of antibodies to a series of bacterial fusion proteins as shown in Fig. 1 was performed as described elsewhere (50).

![Diagram of envelope protein with SU and TM peptides](image)

**Figure 1.** Surface (SU 1 to SU6) and transmembrane (TM 1 to TM3) bacterial fusion products and SU and TM peptides used in the ELISA to detect antibodies against the envelope protein. The top bar represents the envelope protein of FIV, including the leader (L), surface (SU), and transmembrane (TM) proteins. The black boxes represent hypervariable regions in the envelope protein. The different bacterial fusion products and the peptides are indicated as bars.

**VN assays**

VN serum antibodies were determined in two different VN assays. The feline lymphocyte VN assay was based on inhibition of infection of thymocytes with molecularly cloned FIV 19k1 as previously described (242). The CrFK VN assay was based on the inhibition of infection of CrFK 1D10 cells with FIV-AM6c, which is adapted to replicate in these cells. For this assay CrFK 1D10 cells (3.5 x 10^3) were seeded into a 96-well plate in 100 μl of Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. The next day the cells were washed and incubated for 1 h at 37 °C with Polybrene (8 μg/ml) in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. A mixture of equal volumes of the diluted (see above) virus stock of FIV-AM6c, and twofold serial dilutions of heat-inactivated serum were incubated for one hour at 37°C. Then the CrFK cells were washed and incubated with the virus-serum mixture at 37°C. After 24 hours the CrFK cells were washed twice and propagated in Dulbecco modified Eagle medium supplemented with 2% fetal calf serum. After 8 days the culture supernatant was tested for the presence of FIV antigen by ELISA (239). The
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neutralizing antibody titer was expressed as the highest dilution of the serum still preventing FIV antigen production in the culture supernatant.

**Cell associated virus load: infectious center test**

Serially diluted PBMC samples (1 x 10^3, 3 x 10^3, and 1 x 10^4 cells) were prepared from cats before and after challenge as previously described (240). These cells were cocultivated with 10^5 Concanavalin A- and interleukin-2-stimulated PBMC from an SPF cat in eight duplicate wells. After 3 weeks the culture supernatants were tested for the presence of FIV antigen by ELISA. The number of infected cells in the PBMC *in vivo* was calculated from the *in vitro* culture assuming that one infected cell gave rise to antigen production after cocultivation with Concanavalin A- and interleukin-2-stimulated PBMC from an SPF cat, when one or more cultures tested in eight duplicate wells were negative for FIV antigen production.

**Plasma transfer**

A plasma pool was prepared by mixing equal volumes of the plasma samples derived from all the vaccinated cats from groups 1 and 2 at the day of challenge (plasma pool A). A second pool (plasma pool B) was prepared similarly by mixing plasma samples from all the cats of group 6 at the day of challenge. Two groups of four SPF cats, 10 weeks old, weighing between 800 and 1,250 g were used in the transfer experiment. Cats of group A and group B were injected intravenously with 7 ml of plasma pool A and plasma pool B, respectively. Six hours later the cats were challenged by the intramuscular route with 20 CID₉₀ of FIV AM19 as described above. PBMC and plasma samples of the cats were collected at weekly intervals and tested for viraemia by virus isolation and for the development of plasma antibodies against the Gag protein.

**Results**

**Development of FIV-specific plasma antibodies upon vaccination**

All plasma samples collected at the day of challenge were tested in the SU and TM peptide ELISA (Table I, week p.c. 0). All the cats vaccinated with the vGR657 and vGR657x15 iscoms (groups 1 and 2) had developed plasma antibody titers to these peptides, ranging from 200 to 25,000, whereas all the cats vaccinated with Quil A-adjuvanted vGR657x15 (group 3) had developed antibody titers to at least one of the two peptides ranging from 50 to 3,000. Five of six cats vaccinated with
Chapter 4

Table 1. Antibody response against SU and TM peptides in vaccinated cats at different times p.c.*

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* SU peptide ELISA results: no symbol, titer <200; ●, titer between 200 and 1,000; ■, titer between 1,000 and 5,000; ▲, titer between 5,000 and 25,000; ▲▲, titer >25,000. TM peptide ELISA results: no symbol, titer <50; ●, titer between 50 and 300; ▲, titer between 300 and 3,000; ▲▲, titer between 3,000 and 10,000; ▲▲▲, titer >10,000.

the Quil A-adjuvanted bacterial FIV envelope fusion protein (group 4) had also developed serum antibody titers to the TM peptide, ranging from 50 to 3,000, whereas only one animal had developed a titer of 300 to the SU peptide. One cat of
the two control groups, groups 5 and 6, showed an apparently nonspecific plasma antibody titer to the SU and TM peptides, which already existed before the start of the immunization procedure (not shown). Subsequently the same plasma samples were tested in ELISA for antibody titers against the respective bacterial FIV envelope SU and TM fusion proteins (Table 2). Vaccination with the vGR657- and vGR657x15 iscoms (groups 1 and 2) resulted in high plasma antibody responses to all the SU and TM regions tested with the exception of the SU5 and TM1 regions,

Table 2. Levels of antibody against envelope fragments in vaccinated cats at the day of challenge

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* No symbol, OD450 < 0.4; **, OD450 between 0.4 and 0.8; ****, OD450 between 0.8 and 1.2; ******, OD450 >1.2.
against which lower or no antibody levels were detected. Antibody levels induced with the native glycoprotein iscoms (group 1) and the cleavage site-deleted envelope iscoms (group 2) are comparable. The overall OD₄₅₀ values induced with the cleavage site deleted FIV envelope glycoprotein adjuvanted with Quil A (group 3) were lower than those found in the cats of groups 1 and 2. Vaccination with the β-Gal-FIV envelope fusion protein adjuvanted with Quil A failed to induce antibodies against the SU1, SU2, SU4, and SU5 regions. No significant levels of antibody to these fusion proteins were found in the cats of the two control groups (groups 5 and 6).

All the plasma samples collected at the day of challenge were also tested in the two VN antibody assays. None of the samples exhibited VN activity in the feline lymphocyte VN assay (not shown). However, VN antibodies could be demonstrated in cats of groups 1, 2, and 3 in the CrFK VN assay (Fig. 2). The VN titers in cats vaccinated with vGR657 iscoms (group 1) ranged from 160 to 640, with a mean titer of 506. Those of vGR657x15 iscom-vaccinated cats (group 2) ranged from 40 to 640 with a mean titer of 206, which is not significantly different from the values found for group 1. VN plasma antibody titers of cats vaccinated with the Quil A-adjuvanted vGR657x15 protein varied from 10 to 40, with a mean titer of 28, a value which is significantly lower than that found for groups 1 and 2 (Student t test; P<0.05). In cats vaccinated with the β-Gal-FIV envelope fusion protein (group 4) and the cats of the control groups 5 and 6, no VN antibody response could be demonstrated at the day of challenge.

Figure 2. Plasma VN antibody titers of the individual cats of the six different vaccination groups as measured in the CrFK VN assay at the day of challenge. The titers in the individual cats are indicated with different symbols. The mean titer per group is indicated by a bar.
Vaccine development

As expected, none of the cats had developed plasma antibodies to the FIV Gag protein at the day of challenge (Fig. 3).

**Kinetics of FIV-specific plasma antibodies after FIV challenge**

Plasma antibody titers, against the SU and TM peptides of most of the cats immunized with the recombinant FIV envelope proteins (groups 1 to 4) increased 3- to 10-fold within 4 weeks after challenge infection. Within 8 weeks all the cats, including the control animals in groups 5 and 6, had developed anti-SU and anti-TM peptide plasma antibodies (Table 1). At 8 weeks p.c. the SU peptide-specific antibody titers in the cats vaccinated with rVV-expressed FIV envelope glycoprotein were significantly higher than those of the nonvaccinated cats (Table 1; P<0.05).

Plasma antibodies to the FIV Gag protein could be detected 4 weeks after challenge in all the cats vaccinated with rVV-expressed FIV envelope glycoproteins (groups 1, 2, and 3) (Fig. 3). In the cats vaccinated with the β-Gal-FIV envelope fusion protein (group 4) and in the cats of the two control groups, groups 5 and 6, it took about 2 weeks longer before FIV Gag-specific antibodies were detected. Not only was the induction of FIV Gag-specific antibodies in cats of groups 1 to 3 faster, but also the 8 week p.c. plasma titers tended to be higher in these animals (Fig. 3).

![Figure 3. Kinetics of the plasma antibody titer development against the Gag protein, in cats from the different vaccination groups. Mean anti-Gag titers at different times after challenge are presented.](image-url)

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**Cell associated virus load**

Two weeks after infection FIV-infected PBMC were demonstrated in all the cats of groups 1 and 2 and in four of six cats of group 3 (Fig. 4). The numbers of the FIV-infected PBMC varied from 50 to 210, 30 to 1,000, and 0 to 106 per $10^6$ PBMC, respectively. In the other three groups no infected PBMC were demonstrated at this time. Two weeks later, FIV-infected PBMC could be demonstrated in all the cats of all groups. Although a lower average FIV load was observed in cats of group 4 compared with that in the other groups, the differences observed between the groups proved not to be significant.

![Figure 4. Numbers of FIV-infected cells per $10^6$ PBMC in the individual cats (indicated by different symbols) of the different vaccination groups at 2 and 4 weeks p.c. The mean numbers of infected PBMC per group are indicated by a bar.](image)

**Plasma transfer experiment**

Pools of plasma collected at the day of challenge from the cats of groups 1 and 2 (pool A) and from group 6 (pool B) were prepared, and VN titers were determined. The titers, measured in the CrFK VN assay, were 320 and <10, respectively. Two groups of four SPF kittens were inoculated intravenously with 7 ml of plasma pool A or plasma pool B. No plasma VN antibodies could be detected 6 h after the transfer. FIV could be demonstrated in PBMC of one of four cats of group A (20 infected cells per $10^6$ PBMC) but not in PBMC of cats of group B 2 weeks after challenge with 20 CID$_{50}$s of FIV AM19 (Fig. 5). Three weeks after challenge three of four cats of group A and two of four cats of group B exhibited
cell-associated viremia, with significantly higher numbers of FIV-infected cells in the cats of group A (P<0.05). The mean numbers of infected PBMC in the cats from groups A and B at three weeks p.c. were 115 and 6 per $10^6$ PBMC, respectively (Fig. 5). At four weeks p.c. the mean numbers of FIV-infected cells were 111 and 16 per $10^6$ PBMC, respectively. Five and six weeks after challenge FIV could be reisolated from PBMC from all cats of both groups with no clear differences in cell-associated virus load.

Four weeks after challenge, plasma antibodies to the Gag protein could be detected in three of four cats of group A (with ELISA titers ranging from 170 to 800) and in none of the cats of group B (Fig. 6). At 5 weeks p.c. anti-FIV Gag plasma antibodies were demonstrated in all cats of group A (titers from 180 to 2,600) and in only two of four cats from group B (titers from 70 to 100). In the plasma samples collected from all the cats of both groups at 6 and 8 weeks p.c.,
antibodies against the Gag protein were demonstrated. The titers were significantly higher (P<0.05) in the cats of group A than those in the cats of group B from 4 weeks p.c. onward.

![Graph showing kinetics of plasma antibody titer development against the Gag protein in individual cats.](image)

**Figure 6.** Kinetics of plasma antibody titer development against the Gag protein in individual cats, transferred with plasma of pool A (groups 1 and 2) or plasma of pool B (group 6).

**Discussion**

The present paper describes the evaluation of the potential of different candidate FIV vaccines in a vaccination challenge experiment in SPF cats. The most striking finding was that immunization with rVV-expressed FIV glycoproteins, resulting in VN plasma antibodies, led to enhanced FIV infection upon challenge in these cats (groups 1, 2, and 3). This was demonstrated by the more rapid development of PBMC-associated viremia and Gag-specific plasma antibodies. The enhancement effect could be transferred to naive cats with plasma collected from cats immunized with these candidate vaccines. In contrast, cats immunized with an FIV envelope bacterial fusion protein (group 4) did not develop VN plasma antibodies and developed PBMC-associated viremia and Gag-specific plasma antibodies with the same kinetics as the two control groups.

Comparison of the antibody levels induced in the first three groups of cats at the
day of challenge indicated that the immunogenicity of vGR657 and vGR657x15 incorporated into iscoms was higher than that of vGR657x15 presented with Quil A. Both VN plasma antibodies and plasma antibodies to the different SU regions proved to be higher in the first two groups (Fig. 2; Table 2). Plasma antibody levels against most of the SU regions were lower or absent in group 4 at the day of challenge (Table 2). It is not clear why the presence of VN plasma antibodies at the day of challenge correlated not with protective immunity but rather with enhanced susceptibility to FIV infection. It should be noted, however, that the VN activity was demonstrable only in the CrFK VN assay and not in the feline lymphocyte assay. The latter assay should probably be considered more relevant in terms of protective immunity against in vivo FIV infection since, in contrast to feline kidney cells, feline lymphocytes are natural targets for FIV infection. In the transfer experiment it was shown that the enhancement phenomenon could be transferred to naive cats with the plasma of cats of groups 1 and 2. Although not formally proven, this indicated that the enhancement was mediated by FIV-envelope specific antibodies. The mechanism of enhancement proved to be operational at relatively high dilutions: after plasma transfer, no VN antibody activity could be demonstrated in the plasma of the kittens which subsequently showed enhanced FIV infectivity. It has also been shown for HIV-1 that antibody-dependent enhancement (ADE) could still be demonstrated at high dilutions (up to 1:65,000), whereas VN antibody activity can rarely be demonstrated at dilutions higher than 1:1,000 (223). As no reliable systems are available at present to quantify or even detect FIV-enhancing antibodies in vitro, it should be realized that all FIV-neutralizing antibodies found, per definition, should be considered to be the net result of neutralization and enhancement of FIV infectivity measured in vitro.

To date different mechanisms of ADE have been described for lentivirus infections. Complement- and Fc receptor-mediated ADEs have been shown to play a role in HIV-1, HIV-2, and SIV infections (106,107,175,221,257). Recently, another mechanism of ADE was described, in which antibodies neutralized or enhanced HIV-1, dependent on the phenotype of the virus involved (232). From our data it cannot be concluded which mechanism was involved in the observed enhancement of FIV infectivity. Indications for enhanced infectivity after FIV vaccination have been observed before (110). In the experiments of Yamamoto et al. (287) the presence of VN antibodies, demonstrated in an FeT1 cell (feline lymphoid cell line) VN assay, correlated with protective immunity rather than with enhancement of infectivity. This immunity could be transferred to naive animals with plasma of immunized animals (105). The main difference between their vaccination approach and ours is that in our experiments recombinant envelope
proteins were used whereas Yamamoto et al. used inactivated whole virus or virus infected cells as immunogens. Since transfer experiments showed that probably plasma antibodies are involved in the observed mechanisms of enhancement and protection in both series of experiments, it may be speculated that the differences in configuration in which the FIV envelope glycoproteins were presented in both vaccines has led to VN antibodies with different affinities. This may have had direct consequences for their in vivo effects.

Furthermore, the cell substrates used for the production of challenge viruses, may have contributed to the observed differences in outcome of the vaccination experiments. The challenge virus used in our experiments was propagated in primary feline lymphocytes, whereas the challenge viruses in the experiments of Yamamoto et al. were propagated in a feline T cell line (287). Like in the HIV-1 system, T-cell line-adapted FIV may be neutralized more efficiently than virus isolates from primary lymphocyte cultures, which may result in neutralization of the virus in the presence of enhancing antibodies. Furthermore, it may be speculated that, as was recently demonstrated for HIV-1 isolates from one individual (232), T-cell line-adapted FIV is less susceptible to ADE.

Taken together, it should be stressed that the mechanisms leading to the observed phenomenon of FIV enhancement upon vaccination and passive transfer are not fully understood at present. For the development of an effective FIV vaccine, the elucidation of the underlying mechanisms may be crucial. This may also lead to a more rational strategy for the development of HIV-1 vaccines.

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Chapter 5

Summarizing discussion

[Image of a cat playing with a mouse]
In this thesis a series of experiments is described in which newly established molecular clones of FIV derived from a domestic cat with feline AIDS, are used to study the molecular basis of FIV cell tropism, escape from VN antibody, and the potential of candidate FIV vaccines. In chapter 1, it is shown that FIV is a widespread pathogen of cats, which induces an immunodeficiency syndrome similar to AIDS in humans. It has characteristic properties of a lentivirus, including its morphology (199), genome organization (189, 204), chapter 1, 2.3), Mg\(^{2+}\) dependent reverse transcriptase (199), chapter 2.3) and it is antigenically related to ungulate lentiviruses like Visna virus, CAEV and EIAV (57). Based on molecular phylogenetic comparison of the FIV \textit{pol} and \textit{gag} genes with those of other lentiviruses, FIV constitutes a separate cluster in the subfamily Lentivirinae which seems to be more related to the cluster of the ungulate lentiviruses (Visna virus, CAEV, EIAV and BIV) than to that of the primate lentiviruses. It is interesting to note however, that the pathogenesis of FIV infection, eventually resulting in feline AIDS, shows more resemblance to that of the primate lentiviruses (Chapter 1). This may be reminiscent of differences in cell tropism of the respective viruses: The ungulate lentiviruses predominantly infect cells of the macrophage lineage throughout the course of infection, whereas FIV, like the primate lentiviruses, predominantly replicates in cells of both the macrophage and the T cell lineages.

Since FIV is a major pathogen of domestic cats, it should be considered of major veterinary importance. Large numbers of FIV infected domestic cats develop feline AIDS and related disorders, which require veterinary attention. The impact of infections with FIV related viruses on the health of large feral and captive felids is not fully understood at present (190). Although speculations have been made about a predisposing role of infection, with related viruses in outbreaks of infectious diseases in wild felids, like recent outbreaks of canine distemper infections in African lions and large felids in zoos, these speculations have so far not fully been substantiated (95, 96).

Besides the apparent veterinary importance of FIV infection of cats, the infection is also of invaluable importance as an animal model for human AIDS. This was extensively demonstrated in studies described in chapter 2.1 and in similar studies carried out by several other groups. We showed that cats, which were experimentally infected with the Petaluma strain of FIV, developed clear immunological abnormalities long before clinical signs could be detected. These included impaired mitogen and antigen induced proliferative T cell responses, and
inverted CD4/CD8 ratios (chapter 2.1, unpublished observations). Bishop et al. also showed that FIV infection in cats not only affected primary, but also secondary T cell responses in the acute stage of the infection (23,24). These observations of impaired primary and secondary T cell responses, which parallel the findings in HIV seronegative individuals, may underlie the increased susceptibility to opportunistic infections generally observed in cats infected with FIV. Besides the apparent similarities in pathogeneses between the feline and primate lentivirus infections, another advantage of the FIV-cat model is the restricted host range which makes this small animal model relatively cheap and easily accessible for experimentation. It should however be realized that the use of another cellular receptor than the CD4 molecule and the relative genetic distance of FlV from the primate lentiviruses may constitute inherent limitations of this model.

The specificity and kinetics of FIV specific serum antibody responses to structural proteins of the virus, were studied upon natural and experimental infection of domestic cats in chapter 2.3. Like in HIV-1 infection of humans an Env protein specific response was detectable before Gag protein specific antibodies could be demonstrated in cats experimentally infected with FIV. Also in naturally infected cats, it was shown that Env protein specific antibodies were demonstrable in the absence of Gag protein specific antibodies. These data clearly indicated, that diagnostic tests should not solely be based on the demonstration of Gag protein specific serum antibodies.

The generation of a series of molecular clones of FIV directly from the bone marrow of a cat with AIDS, thus obviating an in vitro culturing step, facilitated the study of a number of questions which are difficult to address in HIV infections of humans (Chapter 2.3). Two molecular clones of FIV out of a total of 36 - FIV 19k1 and FIV 19k32 - were selected for these studies. These clones differed in six amino acids of the Env proteins and exhibited similar in vitro growth characteristics. Molecular clones FIV 19k1 and FIV 19k32 were first used to study the molecular basis of differences in cell tropism between different viruses, by making chimeric viruses in which Env protein domains had been exchanged between viruses with different phenotypes: FIV 19k1 could be adapted to replicate in CrFK cells, by exchanging the HV-3 region with that of a biological isolate (FIV AM6c) that had been adapted to replicate in CrFK cells. It was however also shown, that additional substitutions within and outside the Env protein were needed to complete this process of adaptation. One of these additional mutations proved to be a Glu to Lys substitution at amino acid position 409 of the Env protein. It may be speculated that for CrFK cell tropism, a critical basic amino acid sequence of HV-3 is required. The HV-3 region harbours an immunodominant antigenic site,
against which virtually all FIV infected cats develop antibodies (50,56,148). This may explain why serum samples from most FIV infected cats show a broad VN pattern towards a wide variety of CrFK adapted viruses when analyzed in CrFK cells. The broad VN activity is therefore merely a reflection of a dominant anti HV-3 reactivity, leading to steric hindrance of viral entry into CrFK cells, than of in vivo significant VN activity. This was further substantiated by showing that indeed serum samples with a broad VN capacity as demonstrated in the CrFK VN assay, exhibited at the best a narrow homologous virus specific VN activity, when measured in a thymocyte VN assay (Chapter 3.2, 3.3).

The molecular clones FIV19k1 and FIV 19k32 were also used in studies concerning the mechanisms of virus neutralization by feline antibodies in lymphocyte cultures. It was shown that two independent mutations in the Env protein of FIV 19k1 conferred resistance to VN serum antibody of a cat infected with FIV 19k1 (S1422). It was demonstrated that FIV 19k32, which differs in only five amino acids in the SU protein from FIV 19k1, could be made sensitive to VN by S1422 through simultaneous amino acid substitutions in the HV-4 and HV-5 regions between FIV 19k1 and FIV 19k32. The mutations, in HV-4 and HV-5 respectively, were approximately 80 amino acids apart and may well be involved in the formation of a single conformation dependent epitope. In this light it is interesting to note that the two regions in which the relevant mutations have been observed are both located in the C-terminal part of the SU protein of FIV. For HIV-1 it has been demonstrated that especially regions of the C-terminal part of the SU protein contributes to the conformation dependent region that interacts with the CD4 molecule, which is the cellular receptor for HIV (264, for a review, see reference 143). Therefore it may be speculated that the differences in HV-4 and HV-5 involved in the transfer of VN sensitivity between FIV 19k1 and FIV 19k32 directly influence a conformational epitope that interacts with the cellular receptor of FIV.

These studies showed that the availability of a series of closely related molecular clones of FIV, generated from the same cat at the same time point, offers the unique opportunity to study the molecular basis of several phenotypic differences between individual viruses by exchanging genes or gene fragments between viruses with different immunological or cell tropism phenotypes. The immune response and overall pathogenesis can be studied in laboratory cats infected with these viruses and the in vivo phenotypic differences may then be related to molecular differences between these viruses. This will lead to a better understanding of the molecular mechanisms underlying the complex features of lentivirus pathogenesis. So far differences in VN activity toward different molecularly cloned viruses, were only
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studied with one polyclonal antibody, S1422 from one cat (Chapter 3.2, 3.3). Similar studies with monoclonal antibodies, or with serum samples collected at different times after infection of cats with different chimeric viruses, may shed light on kinetics, specificities and biological significance of antibody responses against different sites of the virus. In essentially the same way, kinetics, specificities and biological significance of T cell responses could be studied with chimeric viruses exhibiting different interactions with feline T cells. The *in vitro* CrFK cell tropism studies with different chimeric viruses could be extended with *in vivo* cell tropism studies. To this end cats might be infected with a molecularly cloned virus like FIV 19k1 or FIV 19k32 or selected chimeric viruses, and subsequently different cell types could be monitored with time, for the presence of viruses with a different genetic make up. Similar studies with another molecular clone of FIV have recently been initiated by Beebe et al. (18). In contrast to studies on the pathogenesis of HIV-1 infection in humans, individual animals used in these studies can be infected with well defined molecularly cloned or chimeric viruses, which creates the opportunity to deliberately test for correlation of certain genetic changes with certain pathogenetic features.

The molecularly cloned FIV and a corresponding biological isolate of the virus from the same cat were also used as a basis for a vaccination study in laboratory cats. To this end a virus challenge system was established. Different approaches were considered for the generation of the candidate vaccines to be used. From these, the iscom system for presentation of viral proteins to the immune system, was chosen since it had recently been shown that iscoms not only induce long lasting, high titered biologically active antibody responses, but also potent and long lasting Th and CTL responses (217). A envelope gene obtained by direct PCR amplification carried out on DNA of the bone marrow of cat AM19 was used as the basis for the production of envelope glycoprotein. The envelope glycoproteins were chosen since they have been shown to induce VN antibodies and T cell responses in the cat. Furthermore it has been shown that envelope glycoproteins incorporated into iscoms were capable of inducing protective immunity in the SIV and HIV-2 macaque models (192,193). For the production of the FIV envelope glycoproteins, a rVV expression system was used since this system had been used successfully to produce high levels of native and properly glycosylated proteins of other lentiviruses (217). Only limited amounts of thus produced SU protein were incorporated into iscoms. Therefore it was decided to generate an iscom preparation with a glycoprotein from which the cleavage site between the TM and SU part of the precursor glycoprotein had been deleted. This indeed facilitated the incorporation of the FIV SU protein in the iscom structure. The two thus
constructed iscom preparations with FIV envelope glycoproteins with and without the cleavage site, and the relevant control preparations -cleavage site deleted FIV glycoproteins with QuilA, and a SIV iscom preparation- were used to immunize cats. In addition a non glycosylated bacterial expression product of the same envelope protein, was constructed to serve as an additional candidate vaccine in these studies. The most striking result of these vaccination - challenge experiments was that all the preparations that induced VN antibody detectable in the CrFK VN assay not only failed to induce protection, but even induced enhanced FIV infectivity. The observed enhancement was directly proportional to the levels of thus measured VN antibodies. It is interesting to note that none of the vaccinated cats had developed VN antibodies detectable in the lymphocyte VN assay with FIV 19k1. The cats immunized with the non-glycosylated bacterial expression product, did not develop any VN antibody detectable in either of the VN assays. There may have been a hint of a decreased virus load after challenge in the cats immunized with the bacterial expression product, although possibly due to the low numbers of cats used, the differences were not significant. The enhancement phenomenon observed in the cats vaccinated with the glycosylated proteins of FIV, proved to be mediated by a component present in the plasma of vaccinated cats, since it could be transferred with plasma of vaccinated cats. Studies are ongoing at present to prove that indeed antiviral antibodies induced by vaccination with the respective vaccines, have mediated the observed enhancement. Although indications for enhanced infectivity after vaccination with candidate FIV and EIAV vaccines have been obtained before (110,280), little or no attention has been paid to the possible interference of these antibodies with protective immunity in lentivirus systems. Several mechanisms of ADE have been identified in lentivirus systems in vitro. Whether they all play a role in vivo in FIV and other lentivirus systems is not clear at present. The importance and the mechanisms underlying the observed enhancement phenomenon in vaccination studies, can well be addressed in the FIV cat model, using all the tools that were generated in the course of the studies presented in this thesis.
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Samenvatting

Het feline immunodeficiency virus (FIV) is een virus dat nog geen tien jaar geleden werd ontdekt als de oorzaak van een AIDS-achtig ziektebeeld bij de kat. Net als HIV-infecties bij de mens hebben FIV-infecties bij de kat een persisterend verloop, resulterend in verschillende ziektestadia, die geleidelijk in elkaar overgaan en uiteindelijk leiden tot een dier met een ernstig gestoorde immuunfunctie. FIV komt wereldwijd voor en de studies beschreven in dit proefschrift zijn grotendeels uitgegaan van FIV isolaten en moleculaire klonen verkregen uit Nederlandse katten met feline AIDS. Het virus heeft de karakteristieke eigenschappen van een lentivirus, zoals de morfologie, de genoomorganisatie, en een Mg\(^{2+}\)-afhankelijk reverse transcriptase, en bovendien vertoont het een antigene verwantschap met lentivirussen die bij hoefdieren voorkomen. In de familie van de lentivirussen neemt FIV een aparte plaats in, omdat het qua genoomorganisatie en de antigene verwantschappen meer overeenkomt met hoefdier-lentivirussen, terwijl het ziektebeeld meer gelijkenis vertoont met dat van primate-lentivirussen. Omdat FIV bij katten vrij algemeen voorkomt en een ziektebeeld veroorzaakt dat lijkt op AIDS bij de mens, is dit virus niet alleen van groot veterinair belang, maar heeft infectie met dit virus ook waarde als model voor AIDS bij de mens. Een goed diermodel is vooral van belang om inzicht te verkrijgen in de pathogenese van lentivirusinfecties en in de mogelijkheden voor antivirale therapie en vaccinontwikkeling.

Voor het ontwikkelen van FIV-infectie in de kat als diermodel voor AIDS bij de mens, was het allereerst belangrijk dat technieken, die routinematig worden gebruikt voor de bestudering van HIV-infecties, ook voor het FIV-model werden opgezet. Nadat de standaard virologische en serologische testen voor het FIV-model operationeel waren gemaakt, werd aangevangen met de bestudering van immuunfuncties in geïnfecteerde katten met en zonder klinische symptomen van feline AIDS. Allereerst werd aangetoond dat, net als bij HIV infectie, antistoffen tegen de enveloppeiwitten van het virus eerder aantoonbaar zijn dan antistoffen tegen andere virale eiwitten. In sommige katten bleken tijdens de hele infectie zelfs uitsluitend antistoffen tegen envelopeiwitten aantoonbaar te zijn. Dit heeft als praktische consequentie dat bepaalde diagnostische testen, die uitsluitend op het aantonen van serumantistoffen tegen Gag-eiwitten van FIV zijn gebaseerd, een aanzienlijk percentage vals-negatieve resultaten oplevert. Tevens bleek dat reeds in
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een vroeg stadium van de FIV-infectie, lang voordat klinische symptomen waarnembaar waren, gestoorde immuunfuncties bij geïnfecteerde katten m.b.v. *in vitro* technieken aantoonbaar waren. Ook bij de mens kan al spoedig na HIV infectie met soortgelijke technieken een gestoorde immuunfunctie worden aangetoond.

Moleculaire klonen van FIV werden direct, zonder een *in vitro* kweekstap, geïsoleerd uit beenmergcellen van een huiskat met AIDS. Deze moleculaire klonen maakten studies mogelijk die bij met HIV geïnfecteerde mensen niet kunnen worden uitgevoerd. Uit een totaal van 36 moleculaire klonen werden twee replicatie-competente klonen - FIV 19k1 en FIV 19k32 - geselecteerd. Deze klonen hadden vergelijkbare groeikarakteristieken en hun envelopeiwitten bleken maar in zes aminozuren van elkaar te verschillen. Deze moleculaire klonen bleken net als primaire FIV isolaten, alleen in kattelymfocyten te repliceren. Een aantal primaire virusisolaten, o.a. FIV AM6c, bleek zich na adaptatie ook te kunnen vermeerderen in Crandell feline kidney (CrFK) cellen. Dit is een fibroblast-achtige cellijn, die oorspronkelijk geïsoleerd werd uit een nier van een kat. Na uitwisseling van het hypervariabele envelop gebied, HV-3, van FIV 19k1 met dat van FIV AM6c, bleek ook dit aldus gemodificeerde FIV 19k1 in staat om te repliceren in CrFK cellen. Het duurde echter 52 dagen voordat deze chimere kloon, FIV 19k1PBAM6c, inderdaad dit vermogen om te kunnen repliceren in CrFK cellen vertoonde. Aangetoond werd dat additionele mutaties in en buiten envelopeiwitten nodig waren om FIV te adapteren aan CrFK cellen.

De moleculaire klonen FIV 19k1 en FIV 19k32 zijn ook gebruikt om mechanismen van virusneutralisatie en het ontsnappen aan virusneutraliserende antilichamen te bestuderen. Daartoe werd een SPF kat geïnfecteerd met FIV 19k1. Het serum van deze kat, S1422, afgenomen 22 weken na infectie, bleek de infectiviteit van FIV 19k1 in primaire lymfocyten te neutraliseren. Door FIV 19k1 in aanwezigheid van dit serum in T cellen te laten repliceren, ontstond een zgn. *in vitro* "escape mutant" van FIV 19k1. Bij bestudering van deze mutant bleek dat één enkele substitutiemutaties in regio HV-5 van het oppervlakte-envelopeiwit voldoende was om FIV 19kl te laten ontsnappen aan de virusneutraliserende antistoffen die in serum S1422 aanwezig waren. FIV 19k32 kon niet worden geneutraliseerd door serum S1422. FIV 19k32, dat zoals gezegd in het envelopeiwit in zes aminozuren verschilt van FIV19k1, kon alleen maar gevoelig gemaakt worden voor virusneutraliserende antistoffen in serum S1422, door simultaan substitutiemutaties in HV-4 en HV-5 van het oppervlakte-envelopeiwit aan te brengen. Er kan gespeculeerd worden dat deze aminozuren in HV-4 en HV-5, die ver uit elkaar liggen, betrokken zijn bij een conformationeel epitoot, dat gelegen is in een domein.
Betrokken bij binding van het virus aan de cellulaire receptor.

Het laatste deel van het proefschrift beschrijft de ontwikkeling en evaluatie van kandidaat FIV vaccins onder andere gebaseerd op FIV envelopeiwitten als immunogeen. Voor de presentatie van deze antigenen aan het immuunsysteem werd gekozen voor verschillende adjuveringssystemen, waaronder de iscom presentatievorm. In eerdere studies was gebleken dat iscoms niet alleen een langdurige antistofferespons kunnen opwekken maar tevens goede T-helper cel en cytotoxische T cel responsen kunnen induceren. Gekozen werd voor de productie van envelopeiwitten van FIV met behulp van recombinant vaccinaviirusen, die deze eiwitten in de geglycosyleerde vorm tot expressie brengen. Eiwitten met een hydrofoob gedeelte kunnen relatief eenvoudig ingebouwd worden in iscoms. FIV heeft twee envelopeiwitten: het hydrofiele oppervlakte-eiwit en het hydrofobe transmembraan-eiwit. Deze twee eiwitten worden gevormd uit een "precursor eiwit" dat door enzymen op een specifieke "cleavage site" gesplitst wordt. Door deze "cleavage site" te deleteren werd middels een vaccinaviirus-recombinant een envelopeiwit geproduceerd met een hydrofoob gedeelte dat niet snel gekliefd wordt. Het aldus gemaakte envelopeiwit bleek na zuivering inderdaad efficiënt geïncorporeerd te kunnen worden in iscoms. De FIV envelopeiwitten werden ook in een bacteriëel systeem tot expressie gebracht, waardoor ook een niet-geglycosyleerd eiwit ter beschikking kwam. Gebruikmakend van verschillende adjuveringstechnieken werden katten vervolgens gevaccineerd met vaccins gebaseerd op de aldus geproduceerde expressieproducten. Na vaccinatie konden in alle katten die gevaccineerd waren met FIV envelopeiwitten, antistoffen tegen deze eiwitten worden aangetoond. Alleen in de katten die gevaccineerd waren met geglycosyleerde envelopeiwitten, werden virusneutraliserende antistoffen aangetoond in een test die gebaseerd is op het neutraliseren van FIV-infectie van CrFK cellen. Deze antistoffen waren echter niet in staat FIV-infectie van primaire lymfocyten te neutraliseren. Na challenge-infectie bleek bij de katten die genoemde virusneutraliserende antistoffen hadden ontwikkeld, eerder virus in het bloed aantoonbaar te zijn dan bij katten zonder deze neutraliserende antistoffen. De versnelde infectie kon met plasma van de gevaccineerde dieren worden overgebracht naar naïeve katten. Versnelde infectie na vaccinatie is ook bij andere lentivirusuren waargenomen en deze studies benadrukken nogmaals dat bij de ontwikkeling van vaccins tegen lentivirusuren met dit fenomeen rekening dient te worden gehouden. De mechanismen die aan het fenomeen van versnelde infectie ten grondslag liggen kunnen in het FIV-model waarschijnlijk goed worden bestudeerd. Dit vergroot de waarde van het FIV-model voor pathogenese- en vaccinstudies aan animale en humane lentivirusuren.
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Curriculum vitae

De auteur van dit proefschrift is geboren op 29 september 1955 te Bergen op Zoom geboren, waar in 1972 het MAVO-4 diploma werd behaald. In 1975 werd de studie hbo-a microbiologie aan het Dr. Struycken instituut te Breda afgerond. Na enige jaren te hebben gewerkt op een bacteriologisch (Binnen Gasthuis te Amsterdam, hoofd Prof Zanen) en een immunologisch (Ziekenhuis "de Lichtenberg" te Amersfoort, hoofd Dr. J. Kerckhaert) laboratorium was hij sinds 1982 werkzaam op het RIVM als onderzoeksmedewerker. In 1983 werd aangevangen met de deeltijdstudie biologie aan de Rijks Universiteit Utrecht. Deze studie werd afgerond in 1988 met twee hoofdvakken uitgevoerd bij de vakgroep immunologie en infectieziekten van de faculteit Diergeneeskunde (Hoofd Prof. B. vd Zeijst, begeleider Dr. H. Lenstra) en Laboratorium voor Immunobiologie van het RIVM (Hoofd Prof. A. Osterhaus). Vanaf 1988 was hij werkzaam als wetenschappelijk medewerker bij het laboratorium Immunobiologie van het RIVM. Sinds februari 1995 is hij als wetenschappelijk medewerker in dienst van het Dijkzigt Ziekenhuis en de Erasmus Universiteit te Rotterdam.

Publicaties


Weijer K, R. Van Herwijnen, K. Siebelink, F. UytdeHaag, and A.D.M.E.
Curriculum Vitae


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