FIV Vaccine Development:

A Continuing Challenge

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Table of Contents

1

INTRODUCTION

In part based on:

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Three years after the identification of HIV-1 as the causative agent of an acquired immunodeficiency syndrome (AIDS) in humans in 1983, another member of the Lentiviridae family was discovered in a cattery in Petaluma, California [251]. Originally named feline T-lymphotropic lentivirus, the virus was finally named feline immunodeficiency virus (FIV). Within the Lentiviridae family, FIV is placed between the primate lentiviruses HIV and SIV on the one hand and the ungulate viruses Visna virus, CAEV, EIAV and BIV on the other hand, on the basis of genome structure and organisation (Figures 1A and 2). Although FIV is phylogenetically more distant from HIV-1 than the primate lentiviruses it shares many structural and biochemical characteristics with HIV and given the close resemblance of the pathogeneses of infections with both viruses in their respective hosts, FIV infection of the domestic cat (*Felis catus*) has established itself, in the two decades that have past since its identification, as an excellent small animal model for lentiviral infections in general and lentivirus vaccine development in particular [18,76,383].

Structure and genome organisation

While the overall genomic structure of FIV is similar to that of other lentiviruses, including HIV (for review, see ref. 83), there are important differences [231,256,340] (Figure 1A). The genetic material of lentiviruses is contained within two copies of single strand, positive sense RNA molecules. In the viral particle the genome is encapsidated by a core of nucleocapsids. As in all members of lentiviruses, the genomic blueprint of FIV is characterised by a 5′-*gag-pol-env*-3′ backbone. The *gag* gene encodes the matrix (MA), capsid (CA) and the nucleocapsid (NC) proteins, which are involved in the structural organisation and formation of the viral particle. The *pol* gene encodes the protease (PR), reverse transcriptase (RT), deoxyuridine pyrophosphatase (dUTPase, DU) and integrase (IN) proteins, which are involved in the molecular and biochemical processes required for the replication of the viral genome. The *env* gene encodes the structural envelope glycoprotein, the major viral protein that is present in the viral membrane and a major target for antibody responses. For a schematic representation of the viral particle, see Figure 1B. The viral genome is bookended by identical 5′ and 3′ long terminal repeats (LTR) and three small accessory genes (*vif*, *OrfA* and *rev*) complete the approximately 9.5 kb FIV genetic material.

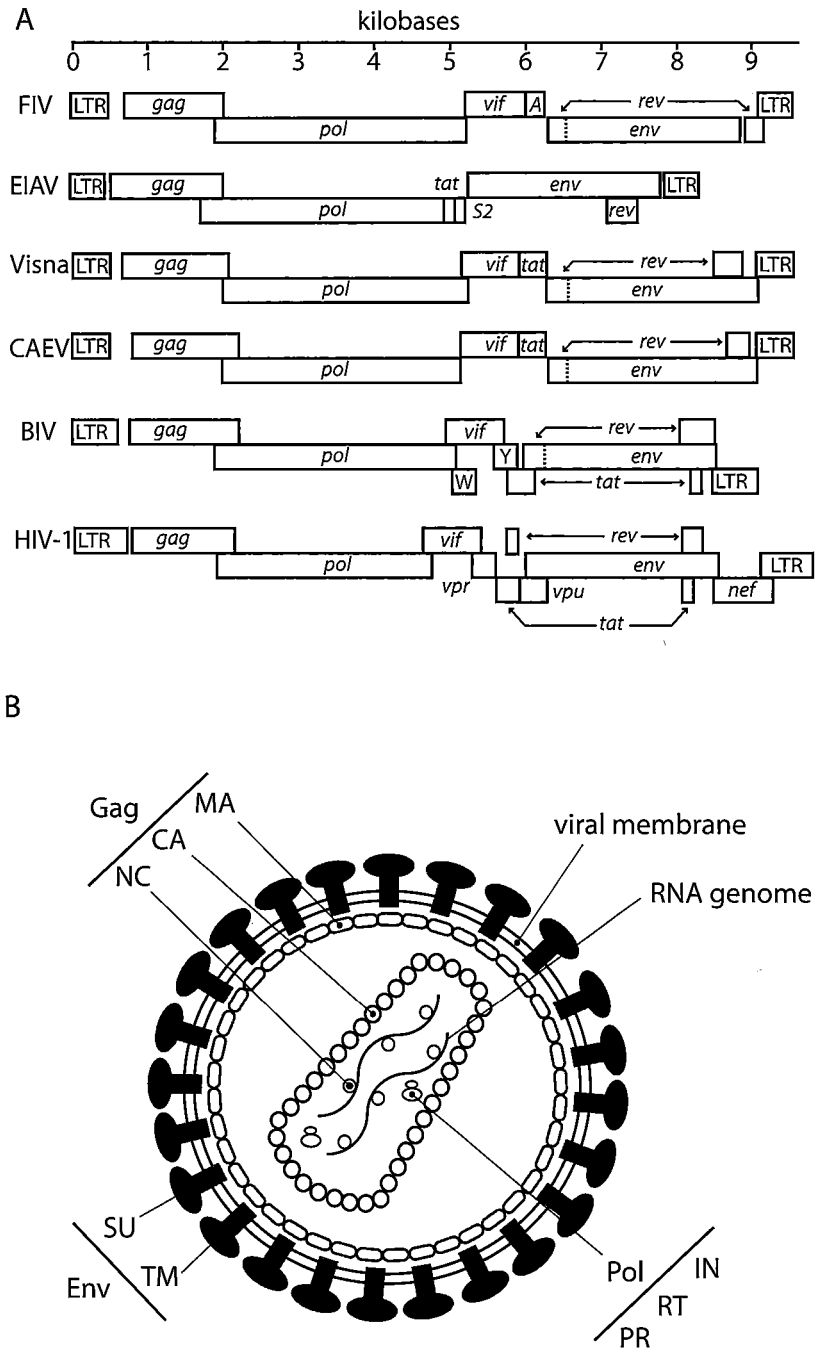

Figure 1. A) Genomic organisation of prototypic members of the Lentiviridae family. B) Schematic representation of the FIV virion and its structural components.

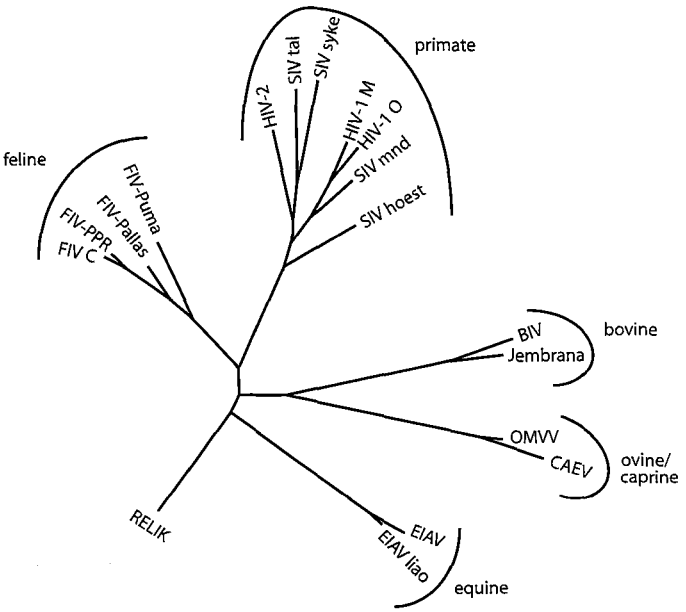

Figure 2. Unrooted phylogenetic tree of the Lentiviridae family, including the recently identified first endogenous lentivirus RELIK (Adapted from Katzourakis et al., Proc. Natl. Acad. Sci. USA (2007) 104(15):6261-5. Used with kind permission from R. Gifford).

Gag

From 5′ to 3′, the *gag* gene encodes the MA, CA and NC proteins. As in the majority of other lentiviruses, the *gag* gene is transcribed into a polyprotein through ribosomal frame-shifting using unspliced viral transcripts as a template yielding Gag- and Gag-Pol polyproteins [217]. The complete, unspliced polyprotein has a role in virus assembly and budding in the late stages of the viral replication cycle. The Gag- and Gag-Pol polyproteins are cleaved by the virally encoded protease (see below) to generate a number of functional proteins, including the *gag*-encoded MA, CA, and NC.

MA, CA, NC

MA directs the polypeptide to the cellular membrane where it plays a crucial role in the incorporation of the envelope glycoprotein into the virion during particle assembly of lentiviruses [70,95,113]. The C-terminal domain of CA is involved in Gag dimerization which is essential for the formation of new virus particles while the N-terminal domain is involved in the formation of the typical morphology of the core of the viral particle [225].

NC contains two zinc finger motifs [81] through which it can bind the viral RNA genome and as such plays an important role in multiple processes of viral assembly including packaging of the genome [265], Gag dimerization [8,286] and compression of the viral genome [21].

In FIV, a P2 protein that is located between Gag and Pol [82] contains a P(S/T)AP domain which is essential for virus budding from the cell [201], similar to the role of the p6 peptide in HIV-1 [208].

Pol

As mentioned above, the FIV Pol polyprotein comprises protease (PR), reverse transcriptase (RT), and integrase (IN) genes. PR is responsible for cleaving the FIV Gag and Gag-Pol polyproteins into separate functional proteins [82]. The nucleotide sequences of the cleavage sites in the viral genome differ between HIV-1 and FIV but they share functional characteristics [82,388]. Since the proteolytic activity of PR is a crucial component in the FIV replication cycle and the processes through which its activity is exerted are highly specific and strictly organised, much effort has been put into the development of specific inhibitors for therapeutic purposes [83].

RT

RT is an RNA- as well as a DNA-dependent DNA polymerase enzyme whose enzymatic properties is Mg^{2+}-dependent. It consists of two active subunits and it is responsible for reverse transcription of the viral RNA genome leading to the formation of a viral DNA genome through an intermediate cDNA molecule [162,380].

IN

After the formation of the double stranded DNA genome, the integrase protein is involved in its integration into the host's genome. Before this can occur, the double-stranded DNA copy of the viral genome has to be transported from the cytoplasm to the nucleus of the infected cell. This occurs in the form of a large nucleoprotein complex, called the preintegration complex (PIC), a stable association of the viral cDNA and the different proteins involved in the integration process [390]. The capacity of both HIV-1 and FIV to productively infect non-dividing cells largely resides in the karyophilic property of the PIC that allows it to cross the nuclear envelope in a way that is independent of the status of the cell-cycle [35,390], although the mechanisms through which this process occurs are not entirely similar for HIV-1 and FIV.

Similar to the ungulate lentiviruses, and unlike the primate lentiviruses, the FIV pol gene contains a gene encoding deoxyuridine pyrophosphatase (DU),

located between RT and IN [82]. DU reduces viral mutations by preventing unwanted incorporation of uracil into cDNA by maintaining a low dUTP:TTP ratio [153]. FIV DU deletion mutant viruses accumulate five- to eightfold more base changes (mostly G to A), compared to wild type FIV during replication in macrophages *in vivo* [174]. DU deletion mutants are not able to productively infect non-dividing cells but DU is dispensable for replication in proliferating cells due to high endogenous levels of DU in these cells [174,373]. Although FIV DU deletion mutant virus is able to infect cats, they replicate to lower levels than wildtype FIV, in particular in the spleen and salivary glands [174].

Env

The *env* gene encodes the envelope glycoprotein, the major viral protein constituent of the viral membrane. Its role in infection is multifactorial in that it is involved in the initial interaction of the virus with the target cell as well as virus entry. As such it is a major target for (virus neutralizing) antibody responses. Besides these evident functions, the envelope glycoprotein is also involved in more complex processes such as apoptosis of activated lymphocytes [100] and neuropathogenesis [270]. It is initially expressed as a precursor protein of about 150kD that is processed by cleavage of a small highly hydrophobic leader sequence upon translocation over the membrane of the endoplasmic reticulum and the subsequent proteolytic cleavage of the remainder into a surface (SU) and a transmembrane (TM) protein. The *env* gene is the most variable of the FIV genes and contains five major consensus variable regions (V1 to V5) in SU and three (V6 to V8) in TM [242,256]. Up to now, four main subtypes have been defined on the basis of *env* sequence variation. These subtypes are not rigid in that various inter-subtype recombinant viruses have been described [9].

Small additional genes

In addition to the already mentioned DU protein, the FIV genome hosts three small additional genes, the functions of which will be briefly discussed below.

Rev

As in HIV, FIV encodes a Rev protein that is involved the transport of unspliced viral RNA molecules or molecules that have been spliced only once from the nucleus into the cytoplasm. The protein contains a nuclear localization signal (NLS), which ensures its translocation to the nucleus. Its coding sequence is contained within two exons that partially overlap with the open reading frame of the envelope glycoprotein. Its role in the transport of viral RNA molecules from the nucleus

of infected cells is mediated by binding of the translocated Rev protein to the Rev Response Element (RRE), which is located just downstream of the *env* open reading frame. It is indispensable for viral replication, both *in vivo* and *in vitro* [164,257,348].

OrfA

Like Tat in HIV-1 and the L protein of Visna virus, FIV also harbours a protein with transactivational properties, OrfA (sometimes referred to as Orf2). It is translated from a bicistronic mRNA molecule that also encodes the downstream exon of Rev [56]. Although functionally similar to Tat, the mechanism through which OrfA stimulates transcription is altogether different [46]. While its NLS directs the protein to the nucleus, rather than acting through a specific "transactivating response region" in the viral LTR, like HIV Tat, OrfA's modest activation profile more resembles that of HIV's accessory Vpr protein [106]. While it is dispensable for replication in adherent cell lines, it is required for productive infection of primary lymphocytes cultures [378]. In vivo replication properties of OrfA deletion mutants demonstrated efficient attenuation and these constructs have subsequently been tested for its potential as a vaccine [260,262]. In addition, a role in virion formation and cytostatic functionality has been described, similar to the HIV Vpr protein [105,106].

Vif

The open reading frame of viral infectivity factor (Vif) is located downstream of *pol*. The protein is derived from spliced viral transcripts containing the RRE [347]. As in HIV, deletion of *vif* coincides with increased mutation rates and severe attenuation both *in vitro* [189,249,302,347] and *in vivo* [152,153,190]. Presence of conserved motif sequences in both HIV and FIV Vif suggest similar modes of action that involve the inhibition of host APOBEC proteins that are responsible for inhibition of replication of HIV and SIV *vif* deletion mutants [190]. Also for *vif* deletion mutants, studies into their capacity as vaccine candidates have been carried out [119].

Epidemiology

While most studies on FIV deal with infection of domestic cats, FIV can also infect a variety of wild felid species. Until now, 27 of 35 evaluated felid species have been shown to contain FIV specific antibodies while PCR analyses have demonstrated lentiviral sequences to be present in the PBMC of 11 species [354]. Infection of wild felid species and their particular FIV strains will not be described here since this thesis deals with FIV infection of domestic cats exclusively. In domestic cats, five clades (A to E) have been defined based on *env* sequences, which can divert up to 30%

between the different clades [9,132,227,259,316], and recently a unique new isolate has been reported [72]. Although there are no official screening programs, over the years it has been reported that the prevalence of FIV in domestic cats ranges from 1% up to about 30%, depending on several factors, including geographic localization and health status of the animal and its population [18]. A large screening study among 18,000 cats in North America showed that 2,5% of the animals contained FIV specific antibodies in plasma [181]. Both prevalence and clade distribution are geographically determined. Whilst clade A is mainly found in north-western Europe and clade B is predominant in southern Europe, in central Europe a mix of both clades is found. In the USA, clade B is most abundant and clade D is largely confined to Japan [159,250,316,326]. Intersubtype recombinations, commonly known to occur frequently in HIV-1 infections, have also been reported for FIV [128,275]. The cat-FIV model provides an excellent animal model system to assess the relevance of different clades with regard to vaccine development (see below). A number of infectious molecular FIV clones have been generated and most have them have been used extensively to study the virus and its infection and for the development of candidate vaccines [15,144,320,340], among them also clone FIV-19k1 [309], the isolate that is used in the majority of the experiments described in this thesis.

Primary and co-receptor usage

In order for a virus to infect its target cell(s), it must interact with cellular components to enable entry across the cellular membrane. For FIV, as for HIV and SIV, entry into target cells is mediated through a sequence of interactions involving a primary and a secondary receptor (or co-receptor). Curiously, for FIV the secondary receptor was identified long before its primary receptor. The explanation for this lies in the fact that FIV strains adapted to replicate in CrFK cells (an adherent cell line that is widely used to study cell tropism and neutralization) can infect these cells without having to interact with a primary receptor. In CrFK cells, FIV infection was shown to be solely dependent on the expression of CXCR4 [142,384,385]. Next, using a CXCR4 antagonist it was shown that also primary FIV isolates used CXCR4 as co-receptor [80,283].

CD134 was subsequently identified to act as the primary receptor for FIV infection [305], after the observation that CXCR4 expression alone was not sufficient to render cells susceptible to infection with certain primary FIV strains [385]. Shimojima et al. identified CD134 through screening of a cDNA library based on a feline IL-2 dependent T cell line for interaction with the virus [304,305]. CD134, the feline homologue of OX40, is a member of the TNFR/NGFR superfamily [305] and was first described as a marker for $CD4^+$ T cell activation in rats [200,247]. Since its discovery as the primary FIV

receptor it was shown to be expressed on IL-2 dependent feline T cell lines [305] as well as on lymph node-derived CD4+ T cells in circulation [158] and it is induced upon ConA stimulation of CD4+ T cells [57]. Collectively, these reports show that identical to the primate lentiviruses, the nature of the primary receptor is responsible for targeting FIV to a distinct subset of lymphocytes: activated CD4+ T cells.

Pathogenesis

FIV infection of cats is associated with a progressive immunodegenerative disease that is similar to that observed in HIV-1 infected humans, termed feline AIDS (FAIDS) [252,319]. Transmission of FIV can occur through transfer of blood but in the field the major route of transmission is biting, with the virus most likely being transferred through the transfer of infected cells. Thus, free ranging male cats that co-inhabit and therefore frequently interact aggressively for territorial space are most commonly infected [53,54,223]. Hence the relatively high prevalence of FIV in countries such as Italy and Japan, where free roaming domestic cats are abundant [12,154]. In addition, vertical transmission, both pre- and postnatally, has been reported [228,229]. Like human AIDS, the development of FAIDS can be divided in a number of sequential episodes, which have been described extensively [18,155]. An acute phase that typically lasts a few weeks is characterised by rapidly increasing viral loads, weight loss, lymphadenopathy and neutropenia. In animals with more severe clinical manifestations, diarrhea, conjuctivitis, dermatitis, gingivitis and respiratory affections can be observed [18]. The ensuing asymptomatic phase is, like in other retrovirus infections, variable in length but in experimental infections immunological and hematological parameters usually do not decline before 2 years after infection, depending both on the virulence of the infecting virus and the health status of the infected animal. This phase is hallmarked by humoral and cellular immune responses that initially control viral replication (although virus can still be isolated from both plasma and PBMC), slowly decreasing CD4+ T cell numbers and, as the name implies, relatively few clinical signs. The continued depletion of CD4+ T cells leads to a progressive immune dysfunction which is furthermore characterized by altered cytokine profiles, loss of T cell proliferative response to mitogen and MHC restricted recall antigens [13,172,350]. In cats experimentally infected with virulent FIV strains ($NCSU_1$, GL8, FC1) the depletion of CD4+ T cells eventually results in an inversion of the CD4:CD8 ratio [144,272,397]. As the infection progresses, the cell tropism of the virus broadens. While activated CD4+ T cells are the main target during acute infection, over the course of infection the virus can also be found in activated B and CD8+ T cells as well as monocytes and macrophages. Since

homologues of the primary receptor CD134 are expressed in these cell populations in humans and mice, this could explain the shift in cell tropism of FIV in cats [387].

The terminal phase of infection is initiated by a period during which persistent generalized lymphadenopathy is seen that is characterised by an enlargement of the lymph nodes. Next, animals undergo a phase during which chronic secondary infections of the oral cavity and upper respiratory tract are often seen that can be caused by numerous agents including viruses, bacteria, fungi and protozoa [18]. Ultimately, surviving animals continue to develop numerous clinical signs of immunodeficiency and increased incidence of opportunistic secondary infections [86]. Apart from secondary infections, cats may also suffer from neurologic [258] and neoplastic disorders [86,98,150]. Lymphoid abnormalities include thymic depletion, lymphoid hyperplasia, plasmacytosis, and terminal lymphoid depletion [349]. In the final stages of the terminal phase, with progressively declining $CD4^+$ T cell numbers, plasma virus loads will increase. Most animals suffer from progressive weight loss and anaemia and also leucopoenia is often diagnosed. If not euthanized, the animals will usually die within 1 year after diagnosis of FAIDS [18], although examples of prolonged survival without disease progression have also been reported [3].

Immune responses

Humoral immune response

Using either ELISA, Western blot and immunoprecipitation methods, antibody responses to the structural FIV proteins from the Gag polyprotein and Env glycoprotein can be detected in experimentally infected animals as early as 2 weeks p.i. [79,93,137]. Whilst Gag specific antibodies are useful as a diagnostic tool since these proteins are relatively conserved within clades, the envelope specific antibodies are believed to play an important role in protective immunity against the virus. Through their interaction with the envelope glycoprotein they are able to neutralize viral infectivity as they may prevent binding to either the primary (CD134) or the secondary receptor (CXCR4). Furthermore, they may opsonize cell free virus, thereby rendering it more susceptible to destruction through phagocytosis or, alternatively, trigger antibody-dependent cellular cytotoxicity responses.

The antibody response directed to the envelope glycoprotein has been studied most extensively. The SU subunit of the envelope glycoprotein contains several virus neutralizing epitopes [19,60,191,310,313] in which single or multiple mutations are involved in escape from virus neutralization [313,368] *in vitro*.

Virus neutralizing (VN) antibody responses have been demonstrated in both experimentally and naturally infected cats [191,351]. It is important to note that VN activity in cat plasma has been measured in two assays that have essential differences.

One is based on the use of a feline fibroblast cell line (CrFK), while in the other feline thymocytes or lymphocytes are used as target cells for infection. For use in the first assay, FIV strains need to be adapted to replicate in CrFK, whilst for the latter assay wild-type viruses are used. It has been shown that infection by CrFK-adapted viruses can be neutralized relatively easy through epitopes located in V3 while wildtype FIV infection of thymocytes is more difficult to neutralize and mainly involves epitopes that are located outside V3 [11,313]. As FIV targets T cells in (naturally) infected cats [17], it is likely that the VN assay based on lymphoid cells most accurately reflects the in vivo situation. Unfortunately, in most studies the CrFK-based assay is used for the detection of VN antibody responses. Therefore, the VN results obtained in the CrFK-based assay should be interpreted with caution. It has also been suggested that the presence of VN antibodies does not necessarily correlate with either virus clearance or disease progression, as virus loads in asymptomatic and symptomatic FIV infected cats do not differ significantly [351,394]. Also, in vaccination and superinfection experiments, the presence or absence of (strain specific) VN antibody responses did not necessarily correlate with the outcome of challenge infection [110,204,209]. On the other hand, the importance of VN antibodies in controlling virus replication has been shown in cats without detectable VN antibody responses that progressed to FAIDS more rapidly than animals with detectable titres. Also, passive transfer studies using sera, plasma or partially purified antibody preparations obtained from vaccinated cats strongly hint towards protective properties of VN antibodies against the virus used for (experimental) infection [131,357]. In contrast, vaccine-induced envelope specific antibody responses have also been shown to correlate with an increased susceptibility to challenge infection with homologous FIV strains [109,161,312]. In one of these studies, such increased susceptibility could be transferred to naïve cats by passive transfer of plasma that was obtained from vaccinated animals on the day of challenge, indicative of a role for antibody responses in this phenomenon [312].

Cellular immune response

As early as two weeks after infection of cats with FIV, cellular immunity mediated by peripheral $CD8^+$ T cells can be demonstrated. Using antigen specific restimulation in vitro, these Gag-specific cytotoxic T lymphocyte (CTL) responses were shown to be MHC class I restricted and remained detectable throughout the acute phase of infection [16]. Although the FIV specific CTL disappeared from circulation during the chronic stages of infection, Gag- as well as Env-specific CTL could still be isolated from lymph nodes at that time point. Other studies showed that precursor CTL could also be isolated from the spleen during the asymptomatic, chronic stages of infection [91,317,318]. In addition to cytotoxic mechanisms of cellular immunity, others have

reported on the induction of $CD8^+$, non-cytotoxic, non-MHC class I restricted anti-FIV responses after infection [34,48,91,102,134]. These $CD8^+$ cells can inhibit viral replication in $CD4^+$ cells in vitro through both contact-dependent and –independent mechanisms. Contact-dependent anti-viral $CD8^+$ cell activity *in vitro* was shown to correlate with decreased provirus loads later in infection [33]. Subsequently it was shown that these $CD8^+$ cells had decreased surface expression of the CD8 beta chain and increased levels of MHC class II [34,102]. Contact-independent antiviral $CD8^+$ activity was shown through the inhibition of FIV replication in vitro by culture supernatants obtained from $CD8^+$ cell cultures originating from FIV infected cats [48,133]. Although these responses do not seem to correlate with increased $CD8^+$ lymphocyte counts after infection, an association with decreased plasma virus loads has been reported [135].

FIV vaccine development: FIV/FAIDS as a model for HIV/AIDS

While studying the pathogenesis of FIV infection of the cat and FIV vaccine development clearly are important from a veterinary point of view, their value to serve as a model for HIV pathogenesis and vaccine development respectively, should not be underestimated: the FIV-cat model is considered to be an excellent small animal model to study the pathogenesis of lentivirus infections, including the development of AIDS, in a natural host [18,383]. FIV, like HIV, can be transmitted via mucosal surfaces, parenterally via e.g. blood transfer, and vertically via prenatal and postnatal routes. The availability of different challenge systems not only provides a useful small animal model to study the importance of different modes of transmission in the pathogenesis of lentivirus infections, but also a model to study the solidity of vaccine induced protection against different routes of infection. Furthermore, although their primary receptors are different, cellular tropisms for FIV and HIV are remarkably similar: both target (activated) $CD4^+$ T cells and monocytes/macrophages but also infect $CD8^+$ T cells and B cells later in infection (see above) [269,294,355]. Progression of infection and development of disease coincide with similar immunological and pathological manifestations (see above) [39,83]. Obviously, for the development and evaluation of intervention strategies like vaccination, this readily accessible model using a non-human pathogen, could provide a wealth of information in a relatively and cheap and easy way: despite having certain distinct molecular features that differ, FIV and HIV share many relevant properties which render the FIV-cat model highly relevant to study pathogenesis and correlates of protective immunity in lentivirus infections.

Vaccine development

Within a few years of its discovery, the first reports on the development of a vaccine against FIV were published. These first studies tested inactivated FIV preparations for their ability to induce protective immunity, either as cell free or infected cell formulations. Since then, numerous vaccine studies have been carried out, using a wide variety of currently available vaccine development approaches. These included live attenuated, live recombinant vector, DNA, recombinant protein and peptide vaccines (Figure 3). Over the years regularly updated review papers have been published by the group of Yamamoto, in which most of the published papers dealing with FIV vaccine development were collected [84,356,357].

Inactivated FIV vaccines consist of either inactivated whole virus (IWV) or inactivated whole FIV infected cell (IWC) preparations. These conventional vaccine approaches have been the most successful in inducing protection from challenge infection in the FIV-cat model to date and ultimately resulted in the registration of the first commercially available FIV vaccine, Fel-O-Vax^{TM} [356]. Initial studies using single-subtype IWV and IWC preparations of FIV_{PET} (a subtype A virus) grown on FL-4 cells (an IL-2 independent feline T cell line) resulted in protection from low dose homologous challenge infection [394]

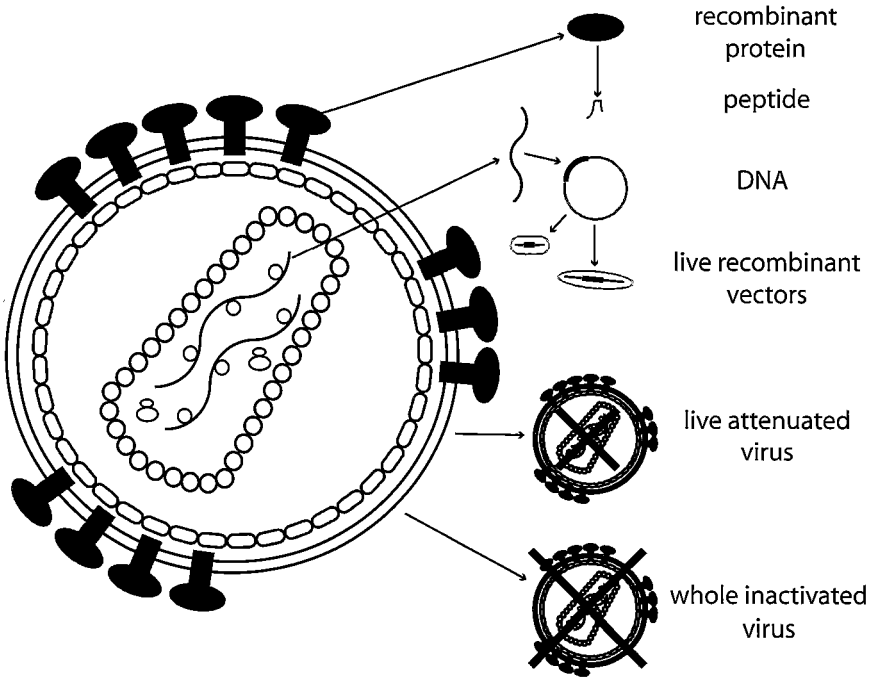

Figure 3. Approaches in FIV vaccine development.

and in protection [395] or reduced viral loads after challenge with heterologous strains of the same subtype [144]. However, these single-subtype vaccine preparations failed to protect from higher challenge doses of heterologous as well as homologous FIV strains [271]. The efficacy of a so-called dual-subtype vaccine, containing two strains from different subtypes (A and D), was tested in the same study and showed significant protection from high dose homologous and heterologous (subtype B) challenge infection [271]. This dual-subtype vaccine was commercially released in the USA in 2002 and has since then been successfully tested against mucosal challenge with homologous and low dose challenge infection of more virulent heterologous strains (subtype B $FIVF_{C1}$) although it failed to protect from a high dose of the same virus (compiled data from ref. 357). In contrast, after challenge infection of Fel-O-Vax™ vaccinated cats with the heterologous subtype B FIV-GL8 strain none of the vaccinees proved to be protected [77]. Furthermore, quantitative analysis of FIV replication kinetics suggested that the vaccine might have primed for increased replication of challenge infection, a phenomenon that has been reported before in a number of FIV vaccination studies that are discussed more elaborately below. In another study using inactivated autologous FIV infected PBMC to vaccinate cats enhanced susceptibility to infection was also observed upon homologous challenge infection [161].

A major problem of vaccines containing whole FIV preparations is of a diagnostic nature: conventional diagnostic routine tests for FIV infection status do not distinguish infected animals from vaccinees inoculated with inactivated virus vaccines [180]. Recently the development of a discriminating ELISA has been reported that, when used in addition to commercial serologic tests, enables distinguishing vaccinated from infected cats [170,182].

Live-attenuated virus preparations have also been explored as FIV vaccine candidates, with limited success. Most of these vaccines consisted of modified full-length proviral DNA constructs where molecular deletions or substitutions were introduced into the viral genome. Partial protection against a low virulence (FIV_{PET}) challenge has been observed in GL8 mutants from which either IN or RT had been deleted when co-immunized with IL-18 expression vectors [75], but the protected animals proved not to be protected from a subsequent homologous GL8 challenge, although virus loads were reduced [75,77]. FIV_{PET} constructs from which the OrfA gene was deleted offered some degree of protection against homologous challenge infection [262]. Vaccination with constructs from which the *vif* gene was deleted induced partial protection in one study [190] but failed in another when engineered to express IFN-γ [119]. Also, constructs in which the antigenic properties of the *env* gene were modified failed to induce protective immunity [31]. An interesting alternative in the use of live attenuated vaccines was described recently, using the "Jennerian" approach, by infecting domestic cats with FIVpco, an isolate normally found in the puma (*Felis*

concolor) that was shown to be attenuated in domestic cats, and prevented $CD4^+$ T cell depletion and immune dysfunction upon FIV infection, even though virus loads were found not to be significantly reduced [342].

Vectored vaccines have also been tested. Using canarypoxvirus constructs engineered to express *env, gag* and *PR* genes induce protection from homologous challenge infection, especially after boosting with IWC vaccine preparations but failed to protect from heterologous challenge infection [341]. Oral immunization with *Listeria monocytogenes* driving the expression of FIV Gag and delivering an Env-expressing DNA construct failed to induce protection from homologous challenge infection but virus loads were reduced in vaccinated cats and virus induced pathology was largely absent [328].

The major risk in using inactivated and/or live-attenuated vaccines against lentivirus infection is incomplete inactivation or under-attenuation, which can result in FIV infection of vaccinees. Also, from the perspective of FIV as an animal model for HIV vaccine development in humans, these types of vaccines are probably not acceptable. Therefore, much effort has been put into the development of subunit vaccines, either in the form of purified viral proteins, recombinant proteins, viral gene DNA vaccines. However, apart from a DNA vaccine that mediated expression of Env from minimal DNA constructs that induced partial but significant protection against homologous challenge infection, these efforts have been unsuccessful (data compiled in [356,357]). Rather, some of these subunit vaccines have been shown to result in enhanced replication of challenge infection [138,281,284,312].

Correlates of protection

Since none of the subunit vaccine approaches has resulted in absolute protection from challenge infection, these studies have therefore not clearly resulted in the delineation of immune responses that correlate with protection from challenge infection. Also, since cats do not clear the virus after natural infection, identifying protective mechanisms by studying immune responses in these animals may only provide limited clues as to the immune responses that an efficacious candidate vaccine should induce. Furthermore, also the successfully employed IWV and IWC vaccines have not yielded clear-cut answers. While in some studies the presence of VN antibody responses on the day of challenge correlated with protection, others have reported on the induction of protection in the absence of VN antibodies. In the cats vaccinated with (live-attenuated proviral) DNA vaccines, an approach known to be rather ineffective in inducing humoral immunity in animals other than mice,

responses in animals that were protected seemed to be associated with cellular immunity, mediated by $CD4^+$ and/or $CD8^+$ T cells [27,75,141,143,190].

A thorough analysis of the immunity induced by the successful IWV and IWC vaccines such as the commercial vaccine has provided some clues as to which responses contribute to vaccine induced protection. Studies in which either passive transfer of plasma or adoptive transfer of T cells was carried out to naïve recipients (MHC class II matched in the case of adoptive transfer of lymphocytes) that were subsequently challenged one day after transfer, strongly suggest the involvement of both humoral and cellular immune responses in the induction of protection from infection. Transfer of either plasma or T cells, both $CD4^+$ and $CD8^+$, resulted in significant protection from both homologous and heterologous challenge infection [357].

Collectively, these studies suggest a significant role for both humoral and cellular arms of the immune system although further research into the specificities of the cellular immune responses in particular is needed for a more rational design of candidate FIV and other lentiviral vaccines. Although initially the development of assays to study these responses was hampered by the lack of reagents specific for the feline system, these reagents are now becoming more readily available, e.g. for the development of IFN-γ ELISPOT assays that have shown to be particularly useful for the identification of potential FIV targets for cellular immunity [65,315]. Furthermore, the development of assays that do not depend on the availability of species-specific reagents [25,359,360] should facilitate the identification of correlates of protective cellular immunity and, thus, the more rational development of new candidate vaccines.

Challenge infection: route and dose

In addition to the fact that several strains of FIV have been used in vaccine research, each with its own specific history (cell culture adapted strains, primary isolates, ex-vivo derived stocks) and virulence, a confounding factor in FIV vaccine research is the lack of a "gold standard" for this purpose. The route, the dose, the strain, and the nature (e.g. cell-associated vs. cell free) of challenge infection should all be taken into consideration. Protection has been observed against intraperitoneal, intravenous, intramuscular, subcutaneous and mucosal challenge infection and the doses used to challenge vaccinated cats range from 5 to 100 CID_{50} [84,356,357]. Protection from high dose mucosal challenge infection may be desirable from the perspective of the FIV-cat model for the development of an HIV vaccine [38,205,328] but protection of cats from FIV infection in a natural setting is more likely to be represented by low dose challenge infection protocols that mimic transfer of cell-associated virus by saliva through biting [75]. Indeed, although the organisation of such trials is elaborate

and requires long-term follow-up, field vaccination studies and trials involving co-mingling of infected cats with uninfected vaccines have shown promising results [169,206]. Matteucci et al. showed that an IWV vaccine that was not protective against a homologous intravenous challenge and partially protective against a mucosal challenge (reduced virus loads), when tested in a private cat shelter setting prevented infection in 12 vaccinees over a two year follow-up period during which 5 out of 14 non-vaccinated controls became FIV positive. Similar results were obtained using Fel-O-VaxTM in a contact challenge experiment [169]. Although complicated in execution and interpretation, these results warrant further exploration for the development and evaluation of veterinary FIV vaccines.

Outline of this thesis

The research described in this thesis expands on previously reported FIV vaccination studies performed in our group. In chapter **2**, a vaccination-challenge experiment is described in which cats are vaccinated with ISCOMs containing different FIV proteins, either in the form of subunits or as whole virus preparations obtained from infected cell cultures, In chapter **3** a vaccination-challenge experiment is described in which cats are vaccinated with ISCOM preparations containing envelope glycoprotein preparations from which regions were deleted that were thought to be responsible for the induction of antibodies involved in vaccine-induced enhancement of infection. In chapter **4**, the first results of FIV subunit vaccination studies using regulatory instead of structural proteins, adjuvanted with ISCOMs, are described. Favourable results obtained with a similar vaccination strategy in the SIV-macaque model prompted us to do so. The latter were based the finding that CTL responses against the early regulatory proteins in HIV-1 were predominantly found in long-term non-progressors. In chapter **5** a similar approach is described, in which DNA and viral vectors were used to deliver these regulatory proteins. Chapter **6** describes intrahost evolution of structural and regulatory protein sequences of FIV. These results not only add to our knowledge of mechanisms behind viral sequence evolution within infected cats but may also aid the rational development of FIV vaccines. Finally the results obtained in the respective studies are summarized and discussed in chapter **7**, with special emphasis on the phenomenon that has played such a crucial role in the scope of the thesis: vaccine induced enhanced susceptibility to virus infection and vaccine induced aberrant viral pathogenesis.

2

FELINE IMMUNODEFICIENCY VIRUS SUBUNIT VACCINES THAT INDUCE VIRUS NEUTRALISING ANTIBODIES BUT NO PROTECTION AGAINST CHALLENGE INFECTION

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Abstract

Three experimental vaccines against feline immunodeficiency virus (FIV), all based on viral antigens presented via immune stimulating complexes (ISCOMs), were tested for their capacity to induce protection in cats from FIV infection. The respective vaccines consisted of FIV propagated in Crandell feline kidney (CrFK) cells (FIV-ISCOMs); FIV-ISCOMs spiked with recombinant vaccinia virus expressed FIV envelope glycoprotein incorporated into ISCOMs (FIV-ISCOMs + vGR657x15-ISCOMs) and vGR657x15 ISCOMs spiked with recombinant FIV Gag protein incorporated into ISCOMs (vGR657x15-ISCOMs+FIV-Gag-ISCOMs). Simian immunodeficiency virus envelope glycoprotein incorporated into ISCOMs, ISCOMs prepared with uninfected CrFK cells, and PBS served as controls. All cats vaccinated with vGR657x15-ISCOMs combined with FIV-ISCOMs or FIV-Gag-ISCOMs developed Env-specifc plasma antibody responses. These antibodies neutralised FIV infection in CrFK cells, but failed to neutralise FIV infection in primary feline thymocytes. FIV-ISCOMs induced poor Env-specific responses and only one out of six cats developed antibodies that neutralised FIV in the CrFK cell based assay. Four weeks after challenge all cats proved to be infected, showing that none of the vaccine preparations provided protection. In contrast, 2 weeks after infection, virus infected peripheral blood mononuclear cells were only observed in cats vaccinated with FIV-ISCOMs + vGR657x15-ISCOMs or CrFK-ISCOMs and to a lesser extent in cats vaccinated with FIV-ISCOMs and vGR657x15-ISCOMs + FIV-Gag-ISCOMs, but not in cats vaccinated with SIV-ISCOMs or PBS. The differences found in cell associated virus loads amongst the respective groups are discussed in the light of antibody mediated enhancement of infectivity and protective effects provided by Gag-specific T cell responses.

Introduction

FIV is a T-lymphotropic lentivirus that causes immunodeficiency syndrome in cats similar to AIDS in humans. Since FIV and HIV share several characteristics and show a similar pathogenesis in their respective hosts, FIV infection of cats proved to be a useful animal model to test antiviral therapies and experimental vaccines against lentiviral infections [308] (for review, see ref. 18). Furthermore, as FIV infection occurs in domestic cats worldwide [253], the development of a vaccine is also of veterinary importance.

Since the discovery of FIV, many attempts have been made to develop a safe and effective vaccine. However, to date only four groups have reported the development of an experimental vaccine capable of protecting cats from challenge infection, albeit only against closely related challenge viruses. These successful vaccines consisted either of fixed FIV infected cells or of whole inactivated virus [23,139,204,394,395]. Two different host cell lines were used to propagate the virus used for vaccination. The first is the FL4 cell line, persistently infected with an American virus strain (FIV/Petaluma) [23,139,394,395], the second is the MBM cell line infected with an Italian FIV isolate (FIV/M2) [204]. The mechanism by which these vaccines induced protective immunity remains unclear. This is further heralded by the observation that both approaches yielded contradictory data concerning possible correlates of protective immunity. Yamamoto et al. and Hosie et al. reported that virus neutralising (VN) antibodies seem to play a major role in the observed protection, as indicated by the observation that most of the protected cats had developed high VN antibody titres against the homologous FIV/Petaluma strain [139,394]. Furthermore, naive cats, passively immunised with pooled sera from vaccinated cats, proved to be protected from subsequent homologous challenge [131]. In contrast, Matteucci et al.found that cats were protected against homologous challenge in the absence of detectable VN serum antibodies [204]. Data collected by Bishop et al. also could not show a role of VN antibodies in the protective responses raised by the FL4/FIV-Petaluma fixed cell vaccine [23]. Interestingly, the latter group suggested that protection was not achieved against latent infection, since the vaccinated cats became FIV-positive when monitored for a longer period of time, i.e. 50 weeks post challenge [23].

Unfortunately, all other attempts to develop FIV vaccines, including experimental subunit vaccines containing either single or combinations of FIV proteins, were of limited success [138,140,195,312]. The majority of these studies used FIV envelope glycoproteins produced using different expression systems and administered with different adjuvants. Although no protection was induced, in some cases a lower cell associated virus load was observed upon challenge infection [140,195]. Also, vaccination

with a synthetic peptide, containing a neutralising antibody inducing epitope, failed to protect cats from challenge infection [192].

Recently, we reported that vaccination of cats with envelope glycoprotein subunit vaccines that induced high titres of VN plasma antibodies accelerated the development of viraemia upon FIV challenge [312]. These VN antibodies could be detected with a Crandell feline kidney (CrFK) cell line adapted virus but not with primary FIV isolates. In addition, the observed enhancement could be transferred to naive cats via plasma pools from the vaccinated animals and was therefore probably mediated by specific antibodies. In the present study we extend these observations by testing a series of experimental ISCOM based FIV subunit vaccines for their ability to induce VN antibody responses detectable against a FIV strain adapted to replicate in CrFK cells and against primary FIV isolates. Subsequently the cats were challenged with a highly homologous virus strain and monitored for the development of viraemia.

Materials and methods

Tissue culture

Feline PBMC and thymocytes were isolated from an 8-week-old SPF cat, as described previously [309]. These cells were stimulated with ConA at a concentration of 5 μg/ml in CM which consists of RPM1 1640 (GIBCO, Gaithersburg, MD) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (2×10^{-5} M) and 10% fetal bovine serum (GIBCO). After 3 days of stimulation the cells were washed and cultured further in CM supplemented with rIL-2 (200 IU/ml) (Eurocetus). The cells were cryopreserved and frozen at -135°C until further use.

A clone of Crandell feline kidney cells (CrFK 1D1O cells) susceptible to infection with FIV, was kindly provided by N. Pedersen and grown under standard conditions [393].

Viruses

FIV AM19 was isolated from PBMC of a naturally infected cat [309]. The virus was subsequently propagated in ConA and rIL-2 stimulated PBMC. After FIV antigen was detected in the culture supernatant by ELISA [307], virus was filtered through a 220 nm pore-size filter, aliquoted and stored at - 135°C. This FIV stock was titrated in viva and subsequently used as challenge stock as described previously [312].

FIV AM6c was initially isolated from PBMC of a naturally infected cat and subsequently adapted to replicate in CrFK lD10 cells and titrated in vitro as previously described [311].

FIV 19kl is a molecular clone, directly derived from bone marrow cells of a naturally infected cat [309]. FIV AM6c and FIV 19kl were used in the two VN assays (see below), amino acid sequence homology of the Env protein of these viruses is 90.1% (K. Siebelink, unpublished results).

Preparation of candidate FIV vaccines

Preparation of the FIV-ISCOMs was carried out with virus obtained from culture supernatant of FIV AM6c infected CrFK cells, which was concentrated with a Provario 100 kDa crossflow filter (J & M Separations). Subsequently, the concentrate was centrifuged for 2 h at 100,000 x g at 10°C on two layers of sucrose of 10% and 50%, respectively. The interphase was harvested and dialysed against PBS for 16 h. With purified FIV virions thus obtained, ISCOMs were prepared as described previously [285]. In brief, the proteins of the virions were solubilised in 2% MEGA- (Boehringer Mannheim) and mixed with the lipids cholesterol and phosphatidylethanolamine (Sigma) and Quil A (Spikoside; ISCOTEC) at a ratio of 1:1:5 (w/w) in the presence of 0.2% MEGA-10. After ultrasonication for 30 seconds the mixture was incubated for 1 h at 37°C followed by dialysis against PBS at room temperature for 16 h and an additional 24 h at 4°C.

vGR657x15-ISCOMs were prepared with recombinant vaccinia virus (rVV) expressed envelope glycoprotein of FIV AM19 from which the cleavage site between the surface and transmembrane protein was deleted (vGR657x15) as previously described [285].

To prepare FIV-Gag-ISCOMs, FIV Gag was expressed with MBP as a fusion protein (MBP-Gag). The gag gene of the 5′ subclone of 19k1 (15) was amplified by PCR, using the following primers (based on the FIV/Petaluma sequence, published by Talbott et al. [340]):

primer 1: 5′-GGAC**GAATTC**ATGGGGGAATGGACCGGGGC-3′;

primer 2: 5′-CCGA**GAATTC**TTACAAATCCAATAGTTTCT-3′.

These primers contain an EcoRI restriction site (bold, underlined) in order to clone the amplified *gag* gene into the EcoRI site of the pMal-CR1 vector (New England Biolabs), thus constructing pMal-Gag by which *gag* is expressed linked to MBP by a sequence sensitive for cleavage by complement factor Xa. pMal-Gag was cotransfected with pUBS520 in *E. coli* BL21-plys to enhance expression of the fusion protein [30]. MBP-Gag was isolated by sonication of the bacteria and subsequently purified by column chromatography using an amylose column. Upon incubation of the fusion protein

on the amylose column and subsequent washing of the column it was digested with factor Xa (New England Biolabs) to isolate the Gag protein. Gag was subsequently coupled to palmitic acid to facilitate incorporation of the Gag protein into ISCOMs, as previously described [277].

Control ISCOMs were prepared with culture supernatant of uninfected CrFK cells that was concentrated with a Provario 100 kDa crossflow filter. Subsequently, 5 mg of total protein was solubilised in 2% MEGA-10 and used for the preparation of ISCOMs as described above.

SIV envelope glycoprotein ISCOMs (SIV-Env-ISCOMs), used as controls in this study, were provided by E. Hulskotte and had been prepared following a procedure similar to that used for the preparation of vGR657x15 ISCOMs [149].

Vaccination procedure and challenge

Five groups of six and one group of five (group 3) SPF cats were vaccinated subcutaneously three times with candidate vaccines and control preparations according to the following schedule: group 1, FIV-ISCOMs; group 2, FIV-ISCOMs+vGR657x15-ISCOMs; group 3, vGR657x15-ISCOMs + FIV-Gag-ISCOMs; group 4, CrFK-ISCOMs; group 5, SIV-Env-ISCOMs; group 6, PBS.

Cats vaccinated with either FIV or SIV-Env-ISCOMs received 10 μg of the respective proteins per dose. Cats vaccinated with FIV-ISCOMs or FIV-Gag-ISCOMs received 10 μg of Gag protein per dose. Protein doses were determined by ELISA as previously described [312].

CrFK-ISCOM vaccinated cats received the same amount of total protein as was present in the FIV-ISCOMs. Cats were vaccinated at 0, 4 and 10 weeks. Two weeks after the last vaccination the cats were challenged intramuscularly with 20 CID_{50} of cell free FIV AM19. Plasma samples and PBMC were collected every 2 weeks, starting 2 weeks prior to challenge infection, as previously described [309].

Serology

Plasma samples of the cats were tested for their reactivity with bacterial fusion proteins containing different regions of the FIV envelope protein by ELISA as previously described [60]. In brief, 96-well microtitre plates were coated overnight at 4°C with the bacterial fusion proteins at 100 ng per well. After washing with PBS/0.05% Tween 20, the plates were blocked with 5% goat serum in PBS/0.05% Tween 20 for 1 h at 37°C, followed by another wash procedure. Hereafter, the cat sera were diluted 1:500 in PBS/0.05% Tween 20/5% goat serum and incubated for 1 h at 37°C. Upon washing, the plates were incubated with horseradish peroxidase labelled goat anti-cat serum (Cappel) in PBS/0.05% Tween 20/5% goat serum for 1 h at 37°C after which

horseradish peroxidase activity was detected. Subsequently, the optical density of the samples was measured at 450 nm in an ELISA reader (Titertek, Multiscan Plus).

Antibodies directed against p24 and p17 Gag proteins were detected with a commercially available ELISA (catalogue number F1002-AB01; European Veterinary Laboratory, Woerden, The Netherlands).

The titres found are expressed as:

OD_{450nm} (plasma) x dilution/3 x OD_{450nm} (negative plasma)

Virus neutralisation assays

VN antibodies present in plasma of the cats were measured in two different VN assays. The first assay is based on the inhibition of infection of CrFK 1D10 cells with an FIV strain adapted to replicate in these cells, FIV AM6c [312]. VN plasma antibody titres were defined as the reciprocal of the highest dilution of a plasma sample still inhibiting FIV replication as measured by the detection of viral antigen in the culture supernatants of the CrFK cells by ELISA [307]. The second assay is based on the inhibition of infection of feline thymocytes with molecular clone FIV 19k1 [310]. VN was considered positive when the RT activity was below two times the background level, measured with uninfected cells.

Cell associated virus load

Cell associated virus load was measured by an infectious centre test, essentially as previously described [312]. In short, serial dilutions of PBMC isolated from the cats ($1{\cdot}10^3$, $3{\cdot}10^3$ and $1{\cdot}10^4$ cells) were prepared and subsequently co-cultured with stimulated PBMC from an SPF cat in eight wells. After 3 weeks culture supernatants were screened for presence of FIV antigen by ELISA and the *in vivo* cell associated virus load was calculated using the assumption that one infected cell gave rise to antigen production in an FIV antigen positive well after co-cultivation with PBMC from an SPF cat when one or more of the eight wells were negative for FIV antigen production.

Statistics

Considering the low number of cats in each group nonparametric tests were used for statistical analysis of the data. For tests between more than two groups the Kruskal-Wallis (K-W) test was applied. For tests between two groups the Mann-Whitney (M-W) test was used. Paired data were analysed with the Wilcoxon test. Since multiple tests were performed the α level of significance was conservatively adjusted to $\alpha = 0.05/3 = 0.017$ (i.e. *P* values below 0.017 are assumed significant).

Results

Development of FIV-specific antibody responses upon vaccination

In cats immunised with FIV-ISCOMs (group 1), no or relatively low antibody responses to most of the different regions of the envelope protein could be demonstrated (Figure 1). In contrast, cats that had received vGR657x15-ISCOMs, both in combination with FIV-ISCOMs and FIV-Gag-ISCOMs (groups 2 and 3), developed a relatively high antibody response to most of these regions, compared with the cats of group 1. In plasma of cats of control groups 4, 5, and 6, virtually no antibodies against these Env regions were detected.

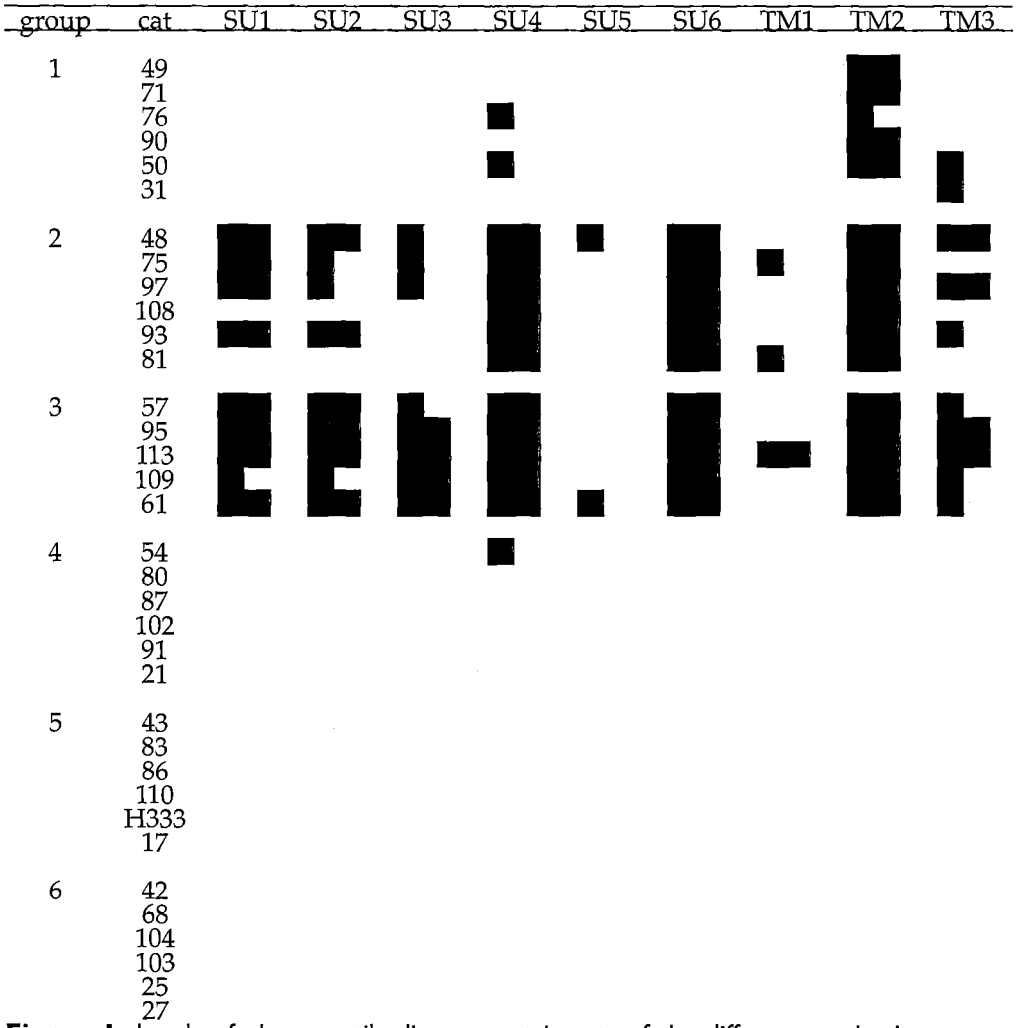

Figure 1. Levels of plasma antibodies present in cats of the different vaccination groups at the day of challenge, against bacterial fusion proteins containing different regions of the FIV envelope glycoprotein 60. Plasma samples were tested at a dilution of 1:500. No symbol, OD450nm < 0.8; ▒, 0.8 < OD450nm < 1.2; ▓, OD450nm > 1.2

In the plasma samples of all cats in groups 2 and 3, collected at the day of challenge, significant VN antibody levels could be demonstrated when tested in the CrFK cell based VN assay ($P \leq 0.004$), with titres ranging from 20 to 640 and a median VN titre of 40 and 160, respectively (Figure 2). In contrast, the cats of group 1, vaccinated with FIV-ISCOMs, did not develop significant VN antibody levels ($P \geq 0.32$), although one cat did develop a VN antibody titre of 160. Moreover, the VN antibody titres of groups 2 and 3 are significantly higher than those of group 1 ($P = 0.019$ and $P = 0.012$, respectively). None of the plasma samples collected at the day of challenge, exhibited VN activity when tested in the thymocyte based VN assay (not shown). None of the cats in the control groups showed any VN activity in either of the assays at the day of challenge (Figure 2).

All cats in groups vaccinated with FIV derived proteins developed significant levels of Gag specific plasma antibodies after two immunisations ($P \leq 0.006$) (Figure 3). At the day of challenge, after three immunizations, cats of group 1, vaccinated with FIV-ISCOMs, exhibited the highest levels of Gag-specific plasma antibodies with a median titre of about 79,000. At this time, the median titres of Gag-specific plasma antibodies in cats of groups 2 and 3, vaccinated with vGR657x15-ISCOMs+FIV-ISCOMs and vGR657x15-ISCOMs+Gag-ISCOMs respectively, were about 40,000 and 57,000. Although the Gag-specific plasma antibody titres of the cats of group 1 tended to be higher than those of groups 2 and 3, titres did not significantly differ compared with each other ($P \geq 0.04$). No Gag-specific plasma antibodies were detected in cats of the three control groups.

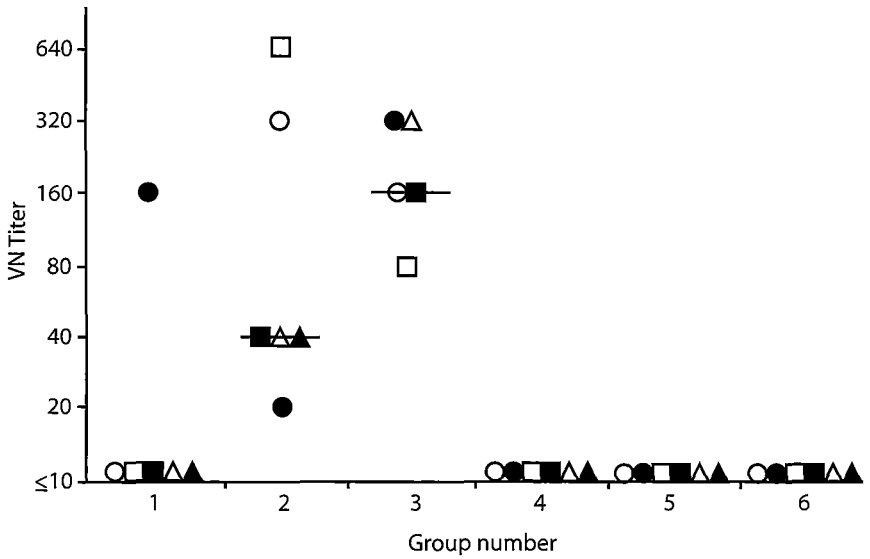

Figure 2. Virus neutralising antibody titres measured at the day of challenge using the CrFK cell based VN assay. Each symbol represents the result of one individual cat. The horizontal bars represent the median VN titre in one group of cats. When the median titre of a group of cats was 0, no horizontal bar is shown

Development of Gag-specific plasma antibody titres after challenge infection

Four weeks after challenge infection with FIV AM19, a clear increase was observed in the Gag specific plasma antibody titres in cats of groups 1 and 2, which were vaccinated with FIV-ISCOMs alone, or in combination with recombinant Env-ISCOMs (median titres of 146,000 and 102,000, respectively) (Figure 3). The increase of Gag specific plasma antibody titres in group 3 was less pronounced (median titre of 60,000), although the lower increase of the Gag specific plasma antibody titres in this group was just at or above the level of significance compared with groups 1 and 2 ($P = 0.018$ and $P = 0.04$, respectively). In cats of the three control groups, Gag-specific plasma antibodies could not be detected until 6 weeks after challenge, with the exception of one cat in group 4 that showed a low titre from week 4 onward.

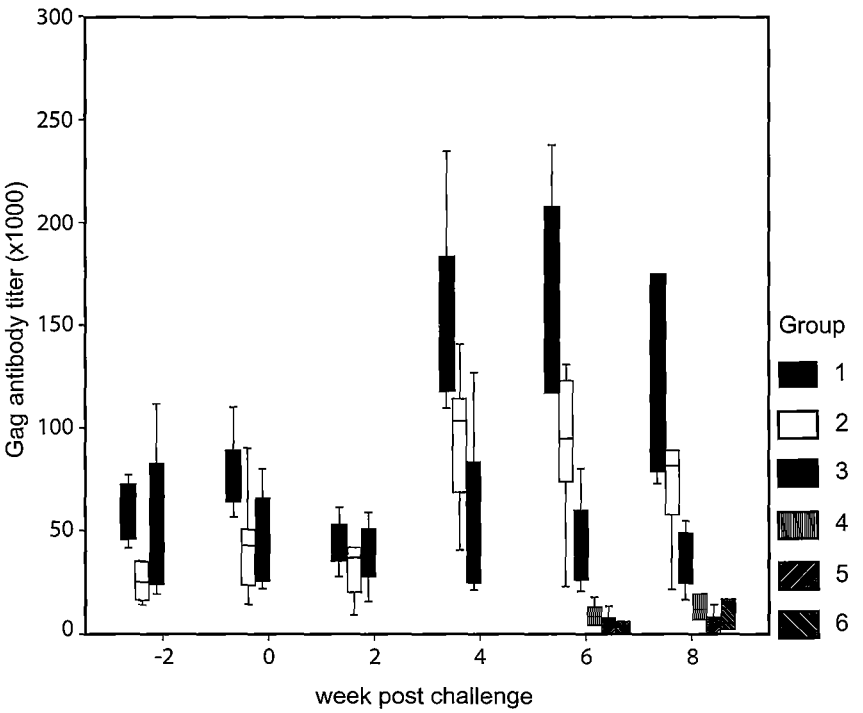

Figure 3. Development of Gag-specific plasma antibodies in the different vaccination groups upon vaccination and subsequent challenge infection. Median Gag-specific plasma antibody titres of each group are presented as horizontal bars within the boxes

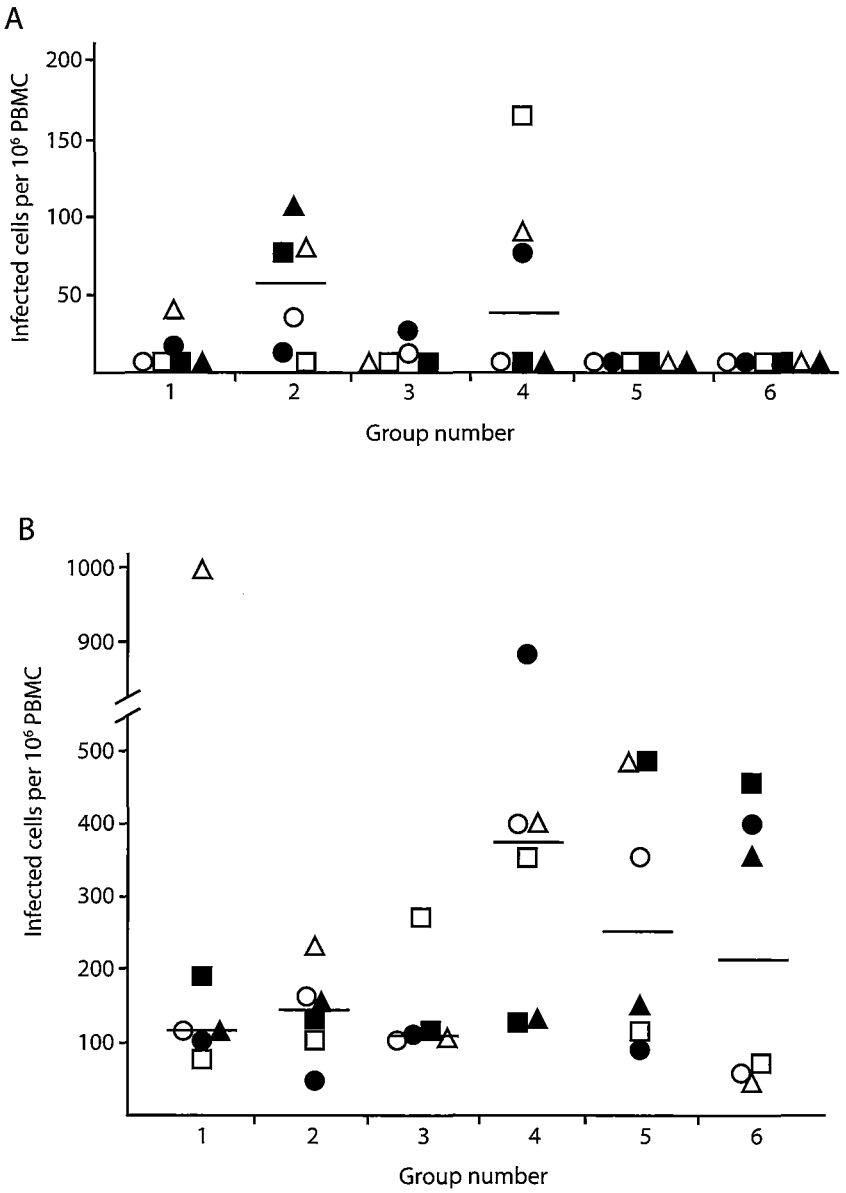

Figure 4. Cell associated virus loads after challenge infection. The number of FIV infected cells per 10^6 PBMC collected from cats at 2 (A) and 4 (B) weeks after challenge were determined in the infectious centre test. Each symbol represents the result of one individual cat, median numbers of infected cells in a group of cats are depicted as a bar. When the median virus load in a group of cats was 0, no horizontal bar is shown

Cell associated virus loads after challenge infection

Two weeks after challenge infection, FIV infected PBMC could be detected in two cats of group 1, with virus loads of 13 and 42 infected cells per 10^6 PBMC, five cats of group 2, with virus loads ranging from 13 to 106 infected cells per 10^6 PBMC, two cats of group 3, with virus loads of I3 and 27 infected cells per 10^6 PBMC, and three cats of control group 4, with virus loads ranging from 76 to 168 infected cells per 10^6 PBMC (Figure 4A). At this time no infected cells could be detected in cats of groups 5 and 6. Statistical analysis revealed that only the cats of group 2 had developed significant numbers of FIV-infected PBMC at this time point compared with control groups 5 and 6 ($P = 0.007$). However, the virus load of cats of group 2 did not differ significantly from that found for the cats of control group 4 ($P = 0.74$).

At 4 weeks after challenge, all cats had become viraemic, with virus loads ranging from 42 to 1,000 infected cells per 10^6 PBMC, and there were no significant differences in the average virus loads between the groups ($P \geq 0.08$) (Figure 4B).

Discussion

In the present study we have shown that, with different ISCOM-based FIV subunit vaccines, high titres of both Gag- and Env-specific plasma antibodies can be induced. The highest levels of envelope specific plasma antibodies were observed in cats of groups 2 and 3, which could be expected since these cats had received vaccine preparations that contained purified envelope glycoprotein incorporated into ISCOMs. In contrast, cats of group 1 had received ISCOMs containing purified FIV derived from infected CrFK cells, in which less envelope glycoprotein may be present.

Although VN activity was induced, albeit only significantly in groups 2 and 3 which were vaccinated with vaccine preparations containing purified vaccinia expressed Env incorporated into ISCOMs, none of the animals proved to be protected from challenge infection with a largely homologous FIV strain. In this context, it is important to note that VN antibodies could only be demonstrated in the CrFK cell based assay, using a FIV strain adapted to replicate in this cell line. In contrast, no VN activity was demonstrated in the primary feline thymocyte based assay, using alargely homologous virus strain (*env* gene sequence homology ≥ 95%) that was not adapted to replicate in CrFK cells. Adaptation to replication in the CrFK cell line apparently alters the sensitivity of FIV to VN antibodies in vitro as we and others noted previously [11,280,312]. In HIV infection of humans, we and others described a similar phenomenon: Antibodies that efficiently neutralise T cell line adapted HIV-1 strains, fail to neutralise or even enhance infectivity of primary HIV-1 isolates in primary lymphoid cell cultures [300,331].

We previously reported the results of studies with FIV subunit vaccines, in which the induction of VN antibodies, only detectable in the CrFK cell based VN assay, predisposed for accelerated viraemia rather than for protection upon FIV challenge [312]. In the experiments reported here, cell associated virus loads, measured two weeks after challenge infection also indicated a tendency toward accelerated viraemia in the cats of group 2, vaccinated with FIV-ISCOMs and vGR657x15-ISCOMs, compared with control groups 5 and 6 ($P = 0.007$). However, three out of six cats of group 4, vaccinated with ISCOMs prepared with the supernatant of uninfected CrFK cells, also proved to be viraemic 2 weeks after challenge, when the SIV-Env-ISCOMs and PBS inoculated groups (5 and 6) were still not viraemic (Figure 4A). Moreover, therespective virus loads of groups 2 and 4 were not significantly different at this time point ($P = 0.74$). Since the cats of group 4 had been immunized with a vaccine preparation devoid of FIV proteins, the accelerated onset of viraemia observed in this group could not be attributed to a FIV specific antibody response. These results imply that, if in this group antibody mediated enhancement had indeed taken place, antibodies to cell derived components in the virus membrane would have caused this phenomenon.

Addition of bacterially expressed Gag protein incorporated into ISCOMs in the vaccine preparation seemed to counteract the enhancing effect induced by vaccination with purified FIV envelope glycoprotein containing vaccines. This is best illustrated by the fact that only two of live cats of group 3, vaccinated with

vGR657x15-ISCOMs and Gag-ISCOMs, had become viraemic 2 weeks after challenge. In a parallel study in which the same challenge infection was used, all six cats vaccinated with vGR657x15-ISCOMs alone had become viraemic at this time and the cell associated virus loads of all the individual cats of that experiment were much higher than those of the two cats of group 3 [312]. It is interesting to note that the two cats in which FIV infected PBMC could be demonstrated, had developed the lowest anti-Gag response of this group at the day of challenge (not shown). Since it cannot be expected that anti-Gag antibodies would have contributed to the observed protective effect, it may be speculated that it was mediated by a Gag-specific T cell response.

Taken together, the differences in outcome between the few series of successful vaccinations versus the large number of unsuccessful FIV vaccination experiments, including those presented in this study, indicate that mechanisms leading to enhanced infectivity may interfere with the induction of protective immunity. Therefore, the rational development of vaccines against lentiviruses including FIV and HIV, should not only focus on the identification of correlates of protective immunity, but also on correlates of vaccine induced enhanced viral infectivity.

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3

ANTIBODIES SPECIFIC FOR THE HYPERVARIABLE REGIONS 3 TO 5 OF THE FIV ENVELOPE GLYCOPROTEIN ARE NOT SOLELY RESPONSIBLE FOR VACCINE-INDUCED ACCELERATION OF CHALLENGE INFECTION IN CATS.

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Abstract

In a previous vaccination study in cats, we reported on accelerated feline immunodeficiency virus (FIV) replication upon challenge in animals vaccinated with a candidate envelope subunit vaccine. Plasma transfer studies as well as antibody profiles in vaccinated cats indicated a causative role for antibodies directed against the hypervariable regions HV3, HV4 and HV5 (HV3-5) of the envelope glycoprotein. The present study was designed to further investigate the contribution of antibodies in envelope vaccine-induced acceleration of FIV infection. To this end, we deleted the regions HV3-5 of the envelope glycoprotein from the original vaccine, thus addressing the contributing role of antibodies directed against these hypervariable regions. Interestingly, this approach did not prevent acceleration of challenge infection. Analysis of the antibody responses in the respective groups suggested that removal of HV3-5 redirected the humoral immune response towards other regions of the envelope glycoprotein, indicating that these regions can also induce antibodies that accelerate virus replication.

Introduction

Since the identification of feline immunodeficiency virus (FIV) in 1986 [251], several candidate FIV vaccines have been developed and evaluated [84,356]. The development of a successful FIV vaccine would not only be of veterinary interest, but could also provide insight into correlates of protective immunity in lentivirus infections. It has been demonstrated that infections with the lentiviruses FIV and HIV-1 share biological and clinical characteristics and exhibit a similar immunopathogenesis which makes FIV infection of cats a valuable animal model for AIDS in a natural host [18,383].

The most promising results with candidate vaccines have been obtained using classical approaches such as inactivated whole virus and infected cell vaccines [88,205,206,271,394,395]. From these studies it has not become clear what the immune correlates of protection were. It has been reported that protection correlated with the presence of virus neutralising antibodies on the day of challenge [394,395], but vaccine-induced protection has also been observed in the absence of virus neutralising antibodies [204]. Candidate FIV subunit vaccines tested to date have failed to induce protective immunity. The majority of these vaccines were based on the use of the FIV envelope glycoprotein (gp130). Vaccines consisted of recombinant bacterial proteins [177,195,312,369], recombinant baculo virus proteins [177,195], recombinant vaccinia virus protein [146,312], peptides representing parts of the envelope protein [87,88,90,192] or recombinant vectors expressing the envelope protein or parts thereof [112,344]. Recently, partial protection was reported using an envelope encoding DNA vaccine co-injected with an IL-12 expression plasmid [27,179].

In contrast, acceleration rather than reduction of FIV replication was observed upon challenge of vaccinated cats in a number of studies [109,138,161,192,281,284,312]. This phenomenon is characterised by an accelerated viremia and sometimes increased viral loads in FIV-specific vaccinated animals compared to mock vaccinated animals. The accelerated virus replication correlated with the presence of envelope specific antibodies at the time of challenge in some studies [109,161,192,312], but not in others [138,281,284]. Hence, the mechanism underlying this enhancement phenomenon has not been fully elucidated. Transfer studies using plasma of vaccinated animals strongly suggested the involvement of plasma associated factors, like virus specific antibodies or cytokines [312]. Moreover, the accelerated viremia also correlated with the presence of antibodies specific for HV3, HV4 and HV5 [312]. We showed that cat antisera raised against FIV molecular clone 19k1 neutralised this virus but enhanced the replication of a closely related molecular FIV clone, 19k32, *in vitro* [309]. Since these two clones differ in their envelope amino acid sequence at only four positions, all located in regions

HV4 and HV5, we hypothesised that antibody responses against these regions are involved in antibody-mediated acceleration of infection.

Here, we report on the in vivo evaluation of a recombinant FIV envelope glycoprotein candidate vaccine from which the regions HV3-5 were deleted. The results show that antibodies directed against HV3-5 are not solely responsible for vaccine-induced acceleration of infection.

Materials & Methods

Cells and viruses

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using Lymphoprep (Axis-Shield, Norway), aliquoted and stored at –135°C. Assays were performed retrospectively to guarantee identical experimental conditions for all samples. After thawing, PBMC were allowed to recover overnight in RPMI (Cambrex Bioscience, Belgium) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), ß-mercaptoethanol (2×10^{-5} M) and 10% foetal bovine serum (Greiner Bio-One, the Netherlands) (=R10F). The following day, the cells were washed and subsequently cultured in R10F supplemented with concanavalin A (4 µg/ml) (Sigma-Aldrich) and recombinant human interleukin-2 (rhuIL-2) (200 IU/ml) (Red Swan Pharma logistics, the Netherlands). After three days, cells were washed and cultured in R10F + rhuIL-2 (200 IU/ml).

Crandel Feline Kidney (CrFK) cells originated from a FIV-susceptible clone, CrFK 1D10, originally provided by N. Pedersen [393] and were cultured in DMEM (Cambrex Bioscience, Belgium) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), ß-mercaptoethanol (2×10^{-5} M) and 10% foetal bovine serum (=D10F).

FIV AM19 was used as the challenge virus, as described previously [312]. This virus was originally isolated from PBMC of a naturally infected cat and an *in vitro* grown virus stock was subsequently titrated *in vivo* [312].

FIV AM6c was used in the CrFK-based virus neutralisation assay (see below). This virus was also isolated from a naturally infected cat and subsequently adapted to replicate on CrFK 1D10 cells [311]. env sequence homology of FIV AM6c and the molecular clone FIV 19k1 is 94.8%.

Vaccines: rVV constructs

The candidate vaccines used in this study were either identical to or derivatives of the vGR657x15 vaccine described previously [285], that was shown to be responsible for vaccine-induced enhancement of challenge infection [312]. vGR657x15 consists

of a vaccinia virus expressed, lentil lectin-sepharose column purified FIV envelope protein. The cleavage site between the surface and transmembrane protein has been deleted through site-directed mutagenesis to facilitate incorporation into immune-stimulating complexes [285]. For clarity, this vaccine is referred to as FIV-Env-iscom.

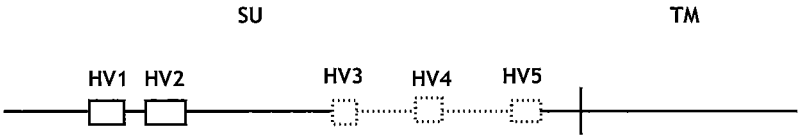

Figure 1. Full length FIV precursor envelope glycoprotein. The regions that were deleted to generate the ΔEnv glycoprotein are dashed. SU = surface subunit, TM = transmembrane subunit, HV = hypervariable region.

A schematic respresentation of the regions deleted from the ΔEnv construct is provided in Figure 1. To generate the FIV-ΔEnv vaccines, the envelope of FIV-19k1 was amplified using two different primer sets: ΔENV1 (5'-GGGTACCTGGAATAACAC) with ΔENV2 (5'-GGTCGACCCACCATCCACATTTTGG; Sal I) and ΔENV3 (5'-GGTCGACGGCATCTTAAGAAATTGG; Sal I) with ΔENV4 (5'-CCTACCCAATCTTCCCAC) generating two fragments: 5'ΔEnv and 3'ΔEnv. These fragments were linked by the introduced Sal I site. This resulted in an envelope sequence missing amino acids 358 – 567 (based on the envelope sequence of FIV-19k1; accession no. M73964; GenBank), which constitute HV3-5. Internal Kpn I sites were used to exchange ΔEnv with the original envelope sequence in pGR657x15, the plasmid originally used to generate vGR657x15 [285]. Subsequent generation of rVV-GR657x15-ΔEnv, production, isolation, purification and incorporation into iscoms was done as described for rVV-GR657x15 [285]. The obtained vaccine is referred to as FIV-ΔEnv-iscom.

Polyclonal rabbit sera raised against bacterial fusion proteins were used to characterise and identify the produced envelope proteins, serum Ra2194 recognises HV4 and serum Ra2279 recognises epitopes located between HV2 and HV3 [60].

Animals, vaccination and challenge.

The SPF cats used in this study were obtained from Harlan (Horst, the Netherlands). An external Ethical Committee approved the protocol used in this study. Twelve animals were randomly distributed into groups: group 1 (n=3) was vaccinated with the original FIV-Env-iscom preparation; group 2 (n=4) was vaccinated with the FIV-ΔEnv-iscom preparation; group 3 (n=5) received PBS (non-vaccinated controls). Originally, group 1 consisted of four animals, however, one animal died during the vaccination scheme due to reasons not related to the experimental set-up

of this study. Cats were inoculated with the respective vaccines at weeks 0, 6 and 10, identical to the previous study [312]. Each vaccine dose contained 1.5 μg envelope protein. Animals were challenged i.m. with 20 CID50 FIV AM19 two weeks after the last vaccination. PBMC and plasma samples were obtained before and 1, 2, 3, 4, 6 and 8 weeks post challenge.

Virus neutralisation assay

A virus neutralisation assay based on the inhibition of FIV AM6c infection of CrFK was performed as described previously [312].

Env-ELISA

Cloning into prokaryotic expression vectors

A modified version of the pThio plasmid (Invitrogen) was used to generate expression vectors containing FIV 19k1 [309] env sequences. The modifications were introduced for detection and purification purposes. Using primers ThioHisfor (5'-GCGTCTAGAATCCCTAACCCTCTCC–3', Xba I) and ThioHisback (5'-CGCGCTGCAGTCAATGGTGATGGTGATGATG–3', Pst I) and pMT/V5-His (Invitrogen) as a template, the V5 epitope and the 6xHis-tag were amplified, after which the PCR product was cloned into Xba I and Pst I digested pThio. The obtained expression vector was named pThio/V5-His. Expression products from this expression vector are thioredoxin fusion proteins, containing a C-terminal V5 epitope for detection purposes and a 6xHis-tag for purification.

Using Pwo Taq-polymerase (Stratagene), env sequences were directly amplified from a 3'-19k1 pUC19 subclone [309]. For the Env-SU construct, the primers were: 5'- CGGGATCCGACCCATTACAAATCCCACTG-3' (Bam HI) and 5'-CGTCTAGATCTTTTTCTTCTAGGTTTATATTC-3' (Xba I). For the Env-TM construct, the primers were: 5'- CGGGATCCGGCATCTTAAGAAATTGGTAT –3' (Bam HI) and 5'- CGTCTAGATTGATTACATCCTAATTCTTGC-3' (Xba I). The obtained amplicons were subsequently digested with Bam HI and Xba I and cloned into Bam HI and Xba I digested pThio/V5-His.

The Env-SU construct incorporates Env19k1 amino acids 322 – 611 (Env19k1, NCBI nucleotide accession no. M73964), containing hypervariable regions (HV) 3, 4 & 5 and flanking regions that are not deleted from the ΔEnv-constructs. The Env-TM construct incorporates Env 19k1 amino acids 567-701, containing the principal immunodominant domain (PID).

The generated plasmids were checked by restriction enzyme analysis and sequencing.

Expression and purification of bacterial recombinant fusion proteins

For expression of the fusion proteins, plasmid DNA was transfected into BL21pLysS bacteria (Stratagene). After culturing a single colony in 10 ml SOB medium with ampicillin for two hours, the bacteria were subsequently cultured in 500 ml SOB/amp until an OD600 of 0.5 – 0.6 was reached. Expression was induced by adding isopropyl ß-D-thiogalactoside (IPTG) (Roche) to a final concentration of 1mM. After 4 hours of induction at 20°C – 37°C, depending on the construct, the bacteria were pelleted by centrifugation at 900 x g for 15 min. and immediately used for purification or frozen o/n at –20°C.

Subsequently, the pelleted bacteria were solubilised o/n in 5 ml lysisbuffer (6M Guanidine·HCl, 0.1M NaH2PO4, 0.01M Tris·HCl, pH8.0) per 500 ml bacterial culture. The resulting lysate was centrifuged for 15 min. at 10,000 x g, 4°C. The supernatant was then incubated with ProBondTM resin (Invitrogen) for 1 hr. at room temperature. After stacking and washing of the columnmaterial, guanidine·HCl was replaced by urea by washing the column with 10 ml washbuffer (8M urea, 0.1M NaH2PO4, 0.01M Tris·HCl, pH = 6.3). After an additional washing step with washbuffer (pH = 5.9), elution was carried out with elutionbuffer (8M urea, 0.1M NaH2PO4, 0.01M Tris·HCl, pH = 4.5). Washing and elution steps were carried out at 4°C. Purification was checked by SDS-PAGE on 15% polyacrylamide gels and Western blot analysis using an anti-V5 monoclonal antibody (Invitrogen). Elution fractions containing the highest concentration of fusion protein were pooled and used in the ELISA.

ELISA

96 well ELISA plates (Corning) were coated with 100 ng of recombinant protein in PBS (Gibco) per well. As a control antigen, a FIV-Orf A-thioredoxin fusion protein was used, expressed and purified as the envelope fusion proteins. After blocking with Meddens reagent (Meddens, Woerden, the Netherlands) supplemented with 0.5% BSA and 0.05% Tween 20 (=ELISA-buffer), the plasma samples were incubated in a 1:100 dilution in ELISA-buffer for 1 hr. at 37°C. Mouse anti-cat IgG (Serotec, 1:300) was used as a 1st conjugate, followed by rabbit anti-mouse IgG*HRP (DAKO, 1:1000). 3, 3', 5, 5'-Tetramethylbenzidine (TMB) in TMB-diluent (Meddens) was used as substrate. The reaction was stopped after 5 minutes by adding an equal volume of 2M H_2SO_4. Background values against the OrfA fusion protein were subtracted from the Env-SU and Env-TM values to obtain specific OD_{450nm} values.

Virus loads

Plasma virus load

Plasma virus loads were determined with a real-time PCR (Taqman) assay according to a protocol described recently [165,178] on an ABIprism 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Primers and probe sequences [165,178] were adapted to the FIV 19k1 sequence when different. Primer concentrations of 15 pmol (300 μM) and 30 pmol (600 μM) were found to be optimal for the forward and reverse primers, respectively. The optimal probe concentration was 10 pmol (200 μM). The real-time PCR was performed using the EZ-core kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The RT-PCR cycling program was initiated with a 2 min 50°C uracyl amperase step, followed by a 30 min 60°C RT step, 5 min 95°C denaturation and 45 cycles of a 2 step PCR of 20 sec 95°C and 1 min 62°C. Data were collected during the annealing step (62°C). A diluted 19k1 virus stock was used in each run to obtain a standard-curve. 190 μl of feline plasma was spiked with a known Phocine Distemper Virus (PDV) stock that was used as an internal control to control for efficiency of RNA isolation (data will be published elsewhere by H.G.M. Niesters). RNA was extracted from plasma (total 200 μl input) and 4 x concentrated (50μl output) with a Magnapure LC Isolation Station (Roche, Almere, The Netherlands), using the Magna Pure LC Total Nucleic acid Isolation kit (Roche, Almere, The Netherlands).

Cell-associated virus load

Provirus loads or cell-associated virus loads were determined using a slightly modified infectious centre test described previously [312]. Briefly, serially diluted PBMC samples obtained after challenge infection were co-cultured in 96-wells U-bottom plates (Greiner Bio-One, the Netherlands) with a Con-A and rhuIL-2 stimulated mixture of PBMC from two SPF cats in ten wells, in threefold. Culture medium containing rhuIL-2 was added weekly to maintain the cultures. After four weeks the culture supernatants were analysed for the presence of FIV antigen by ELISA [307]. The number of infected cells in the PBMC was calculated from the results of the ELISA by assuming that one infected cell gave rise to FIV antigen production after co-cultivation with the stimulated PBMC, when one or more cultures tested in the ten wells were negative for FIV antigen.

Statistical analyses

For statistical analysis of the data between two groups the Mann-Whitney test was used.

Results

Generation of FIV-ΔEnv-iscom

BHK cells were infected in parallel with rVV-GR657x15-Env or rVV-GR657x15-ΔEnv. Two days post infection, crude cell-lysates were prepared and after purification of the FIV glycoproteins iscoms were prepared and quality controlled as described earlier [285]. In addition, iscom preparations were analysed by SDS-PAGE followed by Western blot. As shown in Figure 2A, the FIV-Env-iscom preparation contained an envelope protein with a molecular mass of 130 kDa, confirming the expression of the non-cleaved 688 aa FIV envelope glycoprotein by rVV-vGR657x15, as reported earlier [285]. Deletion of HV3-5 resulted in the expression of a protein with a molecular mass of approximately 60 kDa. Western blot analysis using polyclonal antisera against HV2-3 and HV-4 specific envelope-regions confirmed the successful deletion of HV3-5. As can be seen in Figure 2, the α-HV2-3 serum (A) clearly identified the expression of both the intact envelope and the envelope glycoprotein from which HV3-5 were deleted while with the α-HV-4 serum (B), the envelope glycoprotein was detected but not the envelope glycoprotein from which HV3-5 had been deleted.

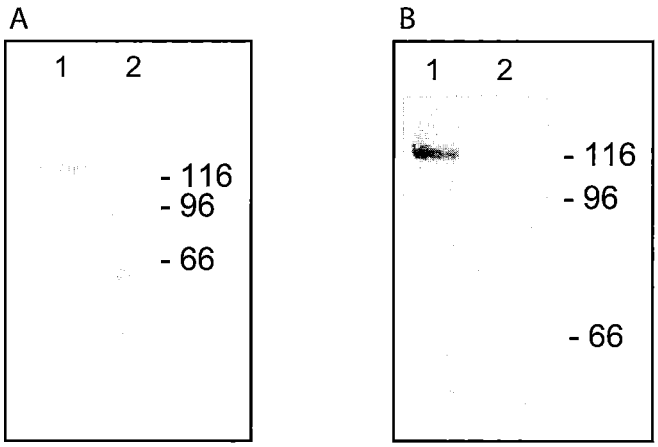

Figure 2. Western blot analysis of candidate vaccine preparations using polyclonal rabbit serum directed against the region between HV2 and HV3 (a) and against HV4 (b) as the primary antibody .Lane 1: FIV-Env-iscom, lane 2: FIV-ΔEnv-iscom. Molecular mass markers are indicated on the right side of each panel.

Vaccine induced immune responses

Virus neutralising antibodies

Cats vaccinated with FIV-Env-iscoms developed virus neutralising antibody (VNA) titres against FIV infection of CrFK cells with titres ranging from 160 to 5120

at the day of challenge (Table 1). The titres were comparable to those measured on the day of challenge in the study by Siebelink et al. in which the same candidate vaccine was used. In plasma samples of control cats and those vaccinated with FIV-ΔEnv-iscoms VNA (titres ≤ 20) were not demonstrated on the day of challenge [312].

Group	Vaccine	Cat no.	Virus neutralization titre (1/x) (weeks post challenge)						
			0	1	2	3	4	6	8
1	FIV-Env	1	160	160	640	5120	10240	2560	640
		2	5120	2560	1280	5120	>40960	10240	5120
		3	320	80	160	80	640	640	1280
2	FIV-ΔEnv	4	<20	<20	<20	80	2560	1280	5120
		5	<20	<20	<20	40	40	10240	5120
		6	<20	<20	<20	40	1280	2560	1280
		7	<20	<20	<20	40	160	10240	10240
3	PBS	8	<20	<20	<20	<20	<20	160	2560
		9	<20	<20	<20	<20	40	2560	10240
		10	<20	<20	<20	<20	<20	80	40960
		11	<20	<20	<20	<20	<20	320	5120
		12	<20	<20	<20	<20	<20	2560	ND

Table 1. Virus neutralization titers. ND = not determined.

Envelope specific antibodies

To study the immunogenicity of the FIV-ΔEnv-iscoms, plasma samples of vaccinated cats were tested in an ELISA for the detection of envelope protein specific antibodies. As shown in Figure 3A, all the cats vaccinated with the FIV-Env-iscoms had developed significant antibody responses against Env-SU by two weeks after the last vaccination ($P = 0.025$, compared to control animals). Two cats vaccinated with FIV-ΔEnv-iscoms mounted an antibody response against Env-SU as well.

The same two animals in group 2 also mounted a significant Env-TM specific antibody response. In contrast, cats from the control group and group 1 failed to develop an antibody response against the transmembrane part of the envelope glycoprotein.

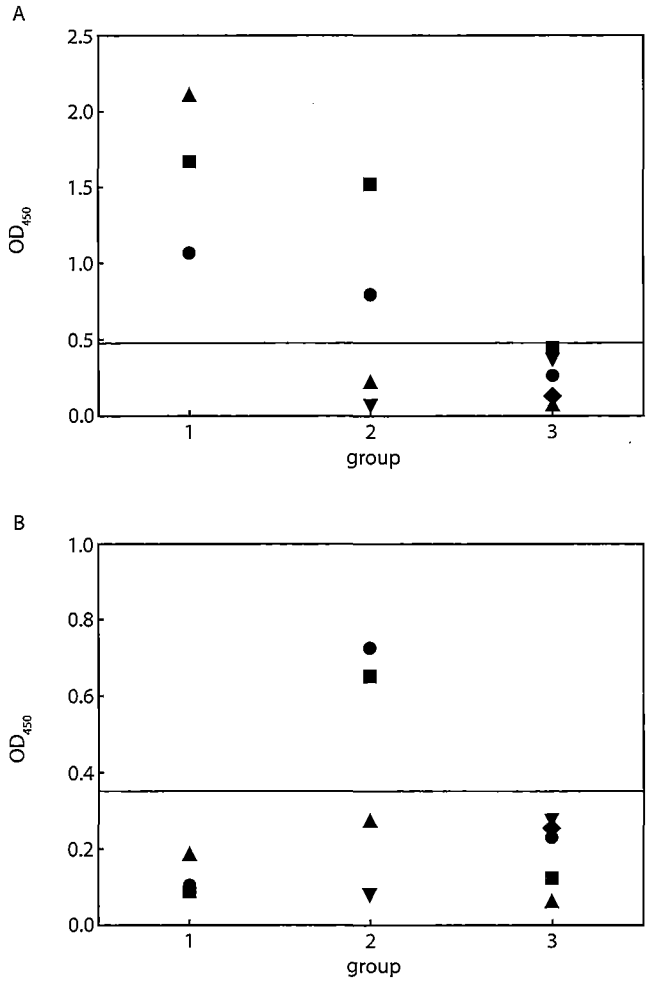

Figure 3. Env-ELISA. Plasma samples obtained on the day of challenge were diluted 1 : 100 and tested by ELISA against 100 ng coated recombinant bacterial fusion protein encoding either the surface (A) or the transmembrane (B) subunit of the envelope protein. Group 1, FIV-Env-iscom; group 2, FIV-DEnviscom; group 3, PBS. Symbols represent individual cats.

Outcome of challenge infection

Plasma virus loads

Plasma virus load kinetics in the respective groups of vaccinated cats were similar irrespective of the vaccine used (Env vs. ΔEnv, Figure 4A). At two weeks post challenge, plasma virus loads were significantly higher in group 2 (median 22,400 copies/ml) compared to control group 3 (median 0, $P = 0.041$). Group 1 (FIV-Env-iscom, median 26,000 copies/ml) also exhibited a trend towards higher virus loads in circulation ($P = 0.167$). Peak virus loads ranged from 60,000 to 4,600,000 copies/ml, with no significant differences between groups. However, envelope protein

vaccinated animals reached peak plasma virus loads one to three weeks earlier than animals in the control group. These plasma virus load kinetics suggest vaccine-induced acceleration of infection in envelope vaccinated animals.

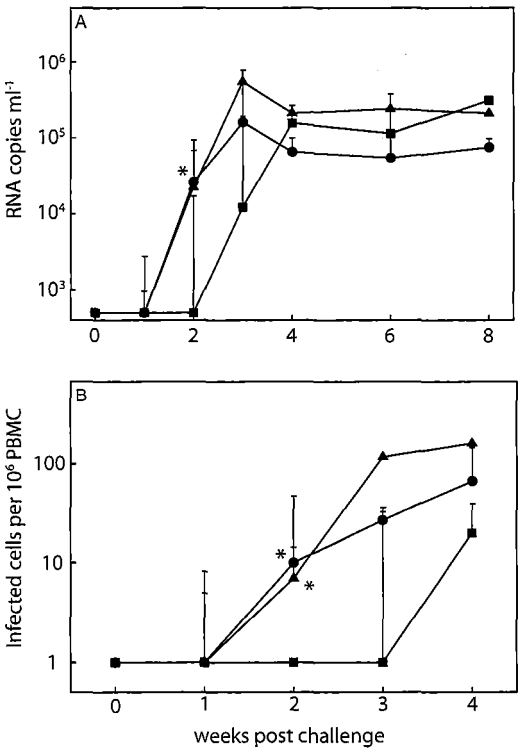

Figure 4. Virus loads. Plasma and PBMC isolated after challenge infection were quantitatively analysed for the presence of FIV particles (A) and provirus (B). ●, Group 1, ▲, group 2, ■, group 3. An asterisk denotes a significant difference compared with control group, $P < 0.05$

Cell-associated virus loads

Accelerated virus replication upon challenge infection was more pronounced when cell-associated virus loads were analysed in the respective groups (Figure 4B): animals vaccinated with the respective candidate envelope vaccines exhibited significantly higher numbers of FIV infected cells at two weeks post challenge (median 30 and 42.5 infected cells/10^6 PBMC, respectively), compared to control animals in which no FIV infected cells were found at this time ($P < 0.05$). Peak provirus loads ranged from 25 to more than 1000 infected cells per 10^6 PBMC, with no significant differences between groups. FIV envelope vaccinated animals showed peak provirus levels two to four weeks earlier than animals in the control group.

Post-challenge virus neutralising antibody kinetics

Upon infection, all cats vaccinated with FIV-Env-iscoms developed an anamnestic antibody response. Virus neutralising antibody titres ≥ 5120 were measured in

individual cats from 3 weeks post challenge onward (Table 1). The FIV-ΔEnv vaccinated cats (group 2) exhibited faster VNA titre kinetics compared to the mock-vaccinated cats (group 3), as 2 out of 4 cats developed VNA titres from 2 weeks post-challenge onward (all cats positive at 3 weeks post challenge), while most mock vaccinated cats did not develop VNA titers until week 6 after challenge.

Discussion

In the present study it was shown that antibodies directed against FIV envelope regions HV3-5 are not solely responsible for vaccine-induced acceleration of challenge infection. We have previously shown that vaccination with an envelope protein-iscom preparation predisposed cats for accelerated viremia upon challenge infection with FIV [312]. Antibodies directed against the envelope protein were considered to be involved in this phenomenon, since naïve cats exhibited an accelerated viremia upon passive transfer with plasma obtained from the vaccinated animals and subsequent virus challenge. The accelerated virus replication correlated with the presence of virus neutralising and HV3-5 specific serum antibodies on the day of challenge [312]. In addition, vaccination of cats with a peptide containing the V3 region of the FIV envelope protein induced enhanced virus replication which correlated with accelerated VNA titre kinetics [192]. To address the role of HV3-5 specific antibodies in vaccine-induced acceleration of infection, we vaccinated cats with iscom candidate vaccines containing either the entire FIV envelope glycoprotein [312] or the envelope glycoprotein from which regions HV3-5 were deleted (FIV-ΔEnv-iscom). It was shown that plasma viral and proviral load kinetics were similar for both the FIV-Env-iscom and the FIV-ΔEnv-iscom vaccinated cats, indicating that antibody responses against HV3-5 were not solely responsible for FIV envelope vaccine-induced acceleration of infection. Passive transfer of post vaccination plasma and subsequent infection of naïve cats could provide supportive evidence that the accelerated virus replication in FIV-ΔEnv-iscom vaccinated cats was antibody mediated as was demonstrated with plasma of cats vaccinated with FIV-Env-iscoms [312]. Since the virus replication kinetics in FIV-ΔEnv-iscom vaccinated cats was similar to that in cats vaccinated with FIV-Env-iscoms, we speculate that also in FIV-ΔEnv-iscom vaccinated cats the accelerated virus replication was mediated by antibodies. In contrast to the FIV-Env-iscom vaccinated groups, enhancement of virus replication was observed in the absence of detectable VNA titres on the day of challenge in FIV-ΔEnv-iscom vaccinated cats (Table 1). The absence of VNA in these animals is most likely explained by the absence of the regions HV3-5 from the vaccine preparation since we and others have shown that neutralisation of FIV infection in vitro is mainly mediated by antibodies directed

against (conformational) epitopes involving regions HV3 [191,193] and/or HV4 and -5 [310,313,368]. Although the HV3-5 deletion may have compromised the conformation of the envelope glycoprotein, the ΔEnv was still immunogenic and env-specific antibodies were detected by ELISA. Since bacterially expressed proteins were used in the ELISA, antibodies against remaining conformational epitopes may have been missed.

Post-challenge serum VNA responses in the FIV-ΔEnv-iscom vaccinated group developed more rapidly than in the control group. This may be caused by the accelerated kinetics of virus replication, by a priming effect of the FIV-ΔEnv-iscom vaccine on the induction of VNA specific for epitopes outside HV3-5 or a combination of both mechanisms. HIV-1 vaccination studies in guinea pigs demonstrated that masking of the envelope V3 region by the introduction of N-linked glycosylation sites and reduction of the net positive charge, resulted in the induction of antibodies specific for the V1 region and with a broader neutralising capacity in vitro than antibodies induced by the original protein [101]. Furthermore, in an attempt to generate immunogenically superior HIV-1 envelope proteins, it has been shown that removal of one or more of the V1, V2 and V3 loops can result in the exposure of normally obscured, conserved neutralisation epitopes [295]. Thus, the antibody response induced by FIV-ΔEnv-iscoms may have been redirected towards regions on the envelope protein other than HV3-5 and have primed for the induction of post-challenge antibodies that can neutralise FIV in vitro but may predispose for accelerated infection in vivo. This is in agreement with the more efficient induction of antibodies specific for epitopes present in the transmembrane region of the envelope glycoprotein observed after vaccination with FIV-ΔEnv-iscoms (see Figure 3B, Env-TM response). As in HIV-1 neutralisation, these epitopes may also be involved in in vitro virus neutralisation of FIV.

It has been suggested that antibodies directed to the principal immunodominant domain (PID), located in the extracellular part of the TM region, are involved in enhancement of HIV-1 infection of cell cultures in vitro [291]. The high similarity between HIV-1 and FIV PID structures [244], suggests that this region may be involved in the induction of antibodies mediating enhancement of FIV infection. However, the observation that immunisation of cats with a peptide containing the FIV PID resulted in reduced virus loads upon challenge infection compared to control animals [282] does not support this option. Collectively, these results point towards major differences between virus neutralisation in vitro and in vivo and it should be stressed that data generated in vitro should be extrapolated cautiously to the in vivo situation. Indeed, the physiological micro-environment has been shown to have a major effect on the infectivity of HIV-1 isolates in PBMC and monocyte derived macrophages [391]. Considering the outcome of this study, epitope deletion as an approach to improve the efficacy of candidate lentiviral vaccines is not likely to eventuate in a successful

vaccine. Although it has been shown for HIV-1 infection in vitro that antibody mediated enhancement by a monoclonal antibody could be abrogated by mutating the targeted epitope [210], this is less likely to occur in the context of a polyclonal immune response in vivo.

In vitro infection experiments have revealed possible mechanisms involved in enhancement of HIV-1 infection besides virus specific antibodies. For example, it has been shown that more efficient virus entry was mediated by components of the complement system and their receptors [29,336]. These mechanisms may also act in vivo. Since in most studies cats are challenged shortly (within two to four weeks) after the last booster vaccination, an additional role for other mechanisms, like immune activation, in vaccine-induced acceleration of infection cannot be excluded [161,281,284]. We have attempted to address the role of immune activation in another group of cats that was vaccinated with FIV-Env-iscoms by challenging these animals twelve weeks after the last booster vaccination instead of two weeks. Despite this adjourned challenge, both proviral and plasma viral load kinetics developed similar to groups 1 and 2 of the current study (data not shown). Both virus neutralising titres as well as envelope specific antibody responses (ELISA) were similar to those described for the FIV-Env-iscom vaccinated animals in the current study. Therefore, it was suspected that antibodies, not vaccine-induced immune activation, were at the basis of the observed accelerated viral replication. However, a control group vaccinated with SIV-Env-iscoms and challenged two weeks after the last booster vaccination exhibited viral load kinetics in between those of the PBS controls and the groups with accelerated viral replication. Since a SIV-Env-iscom vaccinated group challenged twelve weeks after the last booster vaccination was not included, we could not accurately assess the potential involvement of immune activation in this set-up and these groups were therefore not included in this study.

The difference in virus replication kinetics in vaccinated vs. control cats was more pronounced when virus loads were tested as the number of infected cells in the infectious centre test than when plasma loads were tested by real-time PCR (compare Figures 4A and B). This suggests that in the vaccinated cats a more efficient entry of virus into susceptible cells took place. Recent studies suggest that the specific activation of lentivirus specific $CD4^+$ cells results in an increased susceptibility to infection of these cells [71,281,284]. Accordingly, the induction of FIV-specific $CD4^+$ cells by vaccination could have supported a more efficient infection of these cells upon challenge infection, possibly through an increased expression of CXCR4 [385]. However, earlier results showing that a factor responsible for vaccine-induced enhancement of virus replication could be transferred to naïve cats by plasma obtained from cats vaccinated with an FIV-Env-iscom vaccine [312] argue against a role for virus-specific $CD4^+$ T-cells as a single determinant responsible for vaccine-induced enhancement

of virus replication. Still, it cannot be ruled out that multiple vaccine-induced mechanisms are involved in the observed accelerated virus replication.

The present study has shown that vaccine-induced enhancement of FIV challenge infection is not exclusively mediated by antibody responses against epitopes within HV3-5 of the envelope glycoprotein. Since the envelope-iscom vaccine from which these regions were deleted retained its property to induce an antibody response that predisposed for accelerated viremia upon challenge infection, other regions of the envelope glycoprotein may be involved in antibody-mediated acceleration of infection as well. Until those regions are identified, the use of the envelope glycoprotein as a candidate subunit vaccine against FIV infection of cats should be considered with caution.

Acknowledgement

We want to thank Kees Siebelink for his ideas and helpful discussions.

4

Evaluation of ISCOM-adjuvanted subunit vaccines containing recombinant feline immunodeficiency virus Rev, OrfA and Envelope protein in cats

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Abstract

For the development of feline immunodeficiency virus (FIV) vaccines mostly structural proteins have been evaluated for their capacity to induce protective immunity. In the present study, subunit vaccines containing recombinant FIV accessory proteins Rev and OrfA were evaluated in cats. Cats were vaccinated repeatedly with these proteins, adjuvanted with immune stimulating complexes (ISCOM). In addition, cats were vaccinated with bacterially expressed fragments spanning the entire FIV envelope protein, either alone or in combination with the regulatory proteins. Subsequently, the cats were challenged with a homologous FIV strain to assess the level of protective immunity achieved with the respective vaccination regimens. Although the vaccines proved to be immunogenic, vaccinated cats were not protected from infection with FIV.

Introduction

Infection of cats with feline immunodeficiency virus (FIV) has been used as a small animal model for HIV infection in humans. This model proved to be useful for the evaluation of candidate lentiviral vaccines for their capacity to induce protective immunity against infection in a natural host. Many different candidate vaccines have been tested in this model [84], which has resulted in the registration of an adjuvanted whole inactivated virus vaccine in the USA[356] (Fel-O-Vax FIV). This vaccine preparation induced virus specific antibodies and T cell immunity and offered some degree of protection against infection. However, the immune correlates of the observed protection remain to be elucidated [356].

Early vaccination studies in the cat model mainly aimed at the induction of virus neutralising antibodies, using either inactivated whole virus or infected cell preparations or subunit vaccines [84,356]. The majority of these vaccines failed to induce protective immunity and in some cases the induction of antibodies specific for the envelope protein even predisposed for accelerated viremia upon challenge infection [109,161,192,281,312], although this could not be attributed to env-specific antibodies only [147].

It has become clear that for the control of lentivirus replication also cytotoxic T lymphocytes (CTL) are of importance. It was found that in HIV-1 infected patients the magnitude of the CTL response correlated with reduced viral loads [230]. Furthermore, patients with slow disease progression were shown to have broadly reactive cellular immune responses [125,246] in particular directed to the regulatory proteins Rev and Tat [4,358]. Also in SIV-infected macaques, control of viral replication coincided with Rev and Tat specific CTL responses [108]. In addition, vaccine-induced CTL responses against SIV Rev and Tat controlled virus replication more efficiently than those against Gag and Pol [238,330]. With Tat-based vaccines the induction of protective immunity has been achieved in the SIV-macaque model [40], but vaccine failure also has been reported [5,6,184,214,314]. Thus, the performance of vaccines based on the induction of immune responses against lentiviral accessory proteins is still a matter of debate. In the present study subunit vaccines based on the FIV accessory proteins Rev and OrfA, adjuvanted with immune stimulating complexes (ISCOMs), were tested for their capacity to induce protective immune responses in cats. ISCOM was chosen as an adjuvant system since it has been shown that it can induce potent antibody and cellular immune responses [237].

FIV Rev, like its counterpart in HIV-1, is a posttranslational transactivating protein that is indispensable for FIV replication, both in vivo and in vitro [164,257]. FIV OrfA is an accessory protein that is critical for viral replication in lymphocytes and is involved in transactivation of viral transcription, although in a fashion quite dissimilar to that of other lentiviral transactivation proteins such as HIV-1 or Visna Tat [46,334].

In order to obtain sufficient quantities of these proteins for vaccination purposes the Rev and OrfA genes were cloned and expressed as bacterial fusion proteins. Since vaccination with bacterially expressed parts of the FIV envelope induced better protective immunity than vaccination with eukaryotically expressed envelope glycoprotein [312], these were also tested, either alone or in combination with Rev and OrfA, for their ability to protect cats from infection with FIV.

Materials & Methods

Cells

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using Lymphoprep (Axis-Shield, Norway), aliquoted and stored at –135°C. After thawing, PBMC were allowed to recover overnight in RPMI (Cambrex Bioscience, Belgium) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), ß-mercaptoethanol (2×10^{-5} M) and 10% fetal bovine serum (Greiner Bio-One, the Netherlands) (R10F). The following day, the cells were washed and subsequently cultured in R10F containing concanavalin A (4 μg/ml) (Sigma-Aldrich) and recombinant human interleukin-2 (rhuIL-2) (200 IU/ml) (Red Swan pharma logistics, the Netherlands) and used in infectious centre tests to determine provirus loads (see below).

Crandel Feline Kidney (CrFK) cells originated from a FIV-susceptible clone, CrFK 1D10, and were originally provided by N. Pedersen and cultured in DMEM (Cambrex Bioscience, Belgium) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), ß-mercaptoethanol (2×10^{-5} M) and 10% foetal bovine serum (Greiner Bio-One, the Netherlands) (D10F).

Viruses

Molecularly cloned FIV 19k1 [309] was used for the challenge infection of cats. This virus was originally cloned from bone marrow DNA of a naturally infected cat and an *in vitro* grown virus stock was subsequently titrated *in vivo* in parallel with its biological isolate FIV AM19, as described previously [312] (unpublished results). Its amino acid sequence is identical to that of the recombinant proteins used in the vaccine preparations.

FIV AM6c [311] was used in the CrFK-based virus neutralisation assay (see below). This virus was also isolated from a naturally infected cat and subsequently adapted to replicate on CrFK 1D10 cells. *env* sequence identity between FIV AM6c and the molecular clone FIV 19k1 is 94.8%.

Construction of plasmids and expression of bacterial fusionproteins

orfA and *env* genes were amplified by PCR using Pwo Taq polymerase (Stratagene) and a 3'19k1 pUC19 subclone as template [309]. The *rev* gene was amplified by RT-PCR using M-MLV reverse transcriptase (Promega) and mRNA isolated from FIV 19k1 infected cat PBMC as template. All amplicons were cloned into the pThio expression vector (Invitrogen) which was modified to add the V5 epitope and 6xHis-tag for detection and purification purposes, respectively [147]. Since expression of full length Env proved to be difficult, the *env* gene was amplified as five overlapping fragments, which were chosen on basis of hydrophobicity and antigenetic properties of the protein. The resulting fragments (I – V) are shown schematically in Figure 1. Primer sequences used for cloning are available upon request. The identity of the constructs was confirmed by nucleotide sequencing. Optimal incubation temperatures and duration of protein expression were determined for each construct individually as recommended by the manufacturer (Invitrogen).

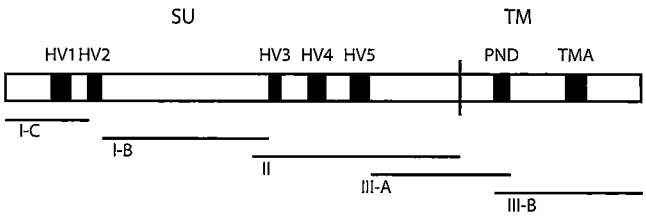

Figure 1. Schematic representation of the envelope protein and the location of the fragments used in the Env-vaccine. In the surface (SU) and the transmembrane (TM) protein, the hypervariable (HV) regions 1 - 5, the principle neutralizing domain (PND) and the transmembrane region (TMA) are indicated. The bacterially expressed protein fragments are designated I - V. The gap between fragments I and II was included in the vaccine formulation as three overlapping peptides (see Materials and Methods for details).

Purification of bacterial recombinant fusionproteins

Since some fusionproteins were expressed in a soluble and others in an insoluble form (inclusion bodies), two different purification procedures were used. The isolation and purification of insoluble fusionproteins has been described previously [147]. For the purification of soluble proteins, bacterial pellets were resuspended in lysisbuffer (50 mM NaH_2PO_4; 300 mM NaCl, pH = 8.0 with 10 mM Imidazole) and sonicated twice for ten minutes on ice (MSE Soniprep 150, Beun de Ronde) followed by freeze/thaw treatment. After centrifugation (15', 10,000 x g at 4°C), supernatants were treated with DNase and RNase (Roche). The preparations were clarified by centrifugation (30', 10,000 x g at 4°C) and filtration (0.45 μm, Millipore) and the recombinant proteins were allowed to bind to a nickel-chelating resin (Invitrogen) through their His-tag. After one hour at room temperature, the resins were stacked in columns, washed with lysisbuffer and washbuffer (lysisbuffer with 20 mM Imidazole). Recombinant proteins were eluted with elutionbuffer (lysisbuffer with 250 mM Imidazole). The purity of

the proteins was confirmed by SDS-PAGE and Western blot analysis using an anti-V5 monoclonal antibody (Invitrogen). Typically, a purity of > 95% was obtained.

Refolding of denatured insoluble proteins

Insoluble fusion proteins (OrfA and all Env fragments) purified under denaturing conditions were allowed to refold prior to their use in vaccine preparations, essentially as described (Current Protocols in Molecular Biology, 1997). A small volume of purified protein in 8M urea was diluted rapidly in excess refolding buffer (50 mM Tris·Cl, pH 7.5; 0.5 M NaCl; 10 mM CHAPS; 2 mM DTT) under continuous stirring at 4°C. Subsequently, the refolded proteins were concentrated using a Centricon-Plus 80 centrifugal filter (Millipore) for 30 – 45' at 1100 x *g* and 4°C. Using SDS-PAGE it was confirmed that soluble proteins were obtained with high yields (data not shown).

Vaccines

For the preparation of ISCOM-based vaccines, the purified bacterially expressed fusionproteins were mixed with lipids/cholesterol and Quadri1+2 in a ratio of 1:1:4, in the presence of 2% MEGA-10 (Sigma). Quadri1+2 is a defined fraction of saponins, purified by HPLC, selected for adjuvant efficacy and minimal toxicity [160], a kind gift from Prof. K. Dalsgaard. After mixing, the preparations were sonicated for 30 min. in an ultrasonic waterbath (Sonicor) and subsequently incubated on a rocker platform for 2 hours at room temperature. Next, the ISCOM preparations were dialysed against PBS using Slide-A-Lyzer (Pierce, Rockford, IL, USA) dialysis membrane-slides and stored at –70°C until use. For vaccination the ISCOM preparations were diluted with PBS to obtain a vaccine dose of 10μg of Rev and OrfA and/or envelope fusion protein.

For the envelope subunit vaccine, the 5 selected fusionproteins were mixed in equimolar amounts. A small stratch of 31 amino acids, located between fragments I and II, could not be expressed. To incorporate this hydrophobic sequence in the vaccine preparation, three peptides were synthesized (EVL, Woerden), overlapping each other and the C- and N-neighbouring expression products by 15 amino acids (NKEKGMTDNVKYGKRCLVGTAAFYLLLAIG, CLVGTAAFYLLLAIGIIIIIRTVDAQVVWRL, IIIIIRTVDAQVVWRLPPLVVPVEESEIIF) and included in the Env-ISCOM vaccine formulation in equimolar amounts.

Animals, vaccination and challenge

SPF cats were purchased from Harlan (Horst, the Netherlands). Twenty-three animals were randomly assigned to four groups. Animals in group 1 (n=5)

were vaccinated with the Rev, OrfA and Envelope preparations, those in group 2 (n=6) with Rev and OrfA, those in group 3 (n=6) with Envelope protein only and group 4 (n=6) was inoculated with the Thioredoxin protein (negative controls). The vaccination and challenge scheme is shown is table 1. Rev and OrfA are relatively small proteins (151 and 78 aa, respectively). In contrast, the FIV envelope glycoprotein is highly immunogenic, containing both B- and T-cell epitopes, some of which are immunodominant [65,89,242,245,280]. Simultaneous vaccination with preparations containing both the accessory proteins and the envelope protein may lead to immune interference [176,248,346]. Therefore, the animals of group 1 were vaccinated sequentially rather than simultaneously. These cats first received two Rev/OrfA doses before Env-ISCOMs were administered. Final booster vaccinations were carried out using both preparations simultaneously.

Ten weeks after the last booster vaccination, the cats were infected intramuscularly with 20 CID_{50} of FIV molecular clone 19k1. Blood sampling was carried out before and at several timepoints after vaccination and at weeks 1, 2, 3, 4, 6, 8, 12 and 23 post infection. Plasma and PBMC were isolated and cryopreserved after each sampling.

Table 1. Experimental groups and timing of vaccination and challenge infection

		time (weeks)						
Group	Vaccines	0	5	8	13	16	36	46
1	R/O + E[a]	R/O	R/O	E	E	R/O + E	R/O + E	Ch.
2	R/O	R/O	--	R/O	--	R/O	R/O	
3	E	E	--	E	--	E	E	
4	mock	mock	mock	mock	mock	mock	mock	

[a] R = Rev, O= Orf-A, E = Envelope., Ch. = challenge infection.

ELISA for the detection of Rev and OrfA specific antibodies

For use in ELISA recombinant Rev and OrfA fused to maltose binding protein (MBP) were obtained. To this end the *rev* and *orfA* genes were cloned into pMalC (New England Biolabs). Induction of expression and purification of the fusion proteins was essentially performed as described previously [372].

MBP-Rev, MBP-OrfA or MBP were used to coat ELISA plates (Greiner) at 100ng in PBS per well. Cat serum samples were tested at a dilution of 1:800 (Rev) or 1:400 (OrfA) in dilution reagent (Meddens) for one hour at 37°C, which gave the best signal to background ratio. A monoclonal antibody against cat IgG (Serotec) was used as secondary antibody, which was detected with a horseradish peroxidase labelled rabbit anti-mouse IgG antibody preparation. After each incubation the plates were washed with PBS containing 0.05% Tween 20. The plates were developed by adding tetramethylbenzidine (TMB) substrate (Meddens) to the wells. Reactions were stopped by adding an equal volume of 1 M H_2SO_4. The optical density was

determined at 450nm and the values obtained with MBP were substracted from those obtained with MBP-Rev and MBP-OrfA.

Virus neutralising antibodies

Virus neutralising (VN) antibody titers in plasma were determined as described previously [312].

Hematology

$CD4^+$ and $CD8^+$ lymphocyte counts were determined on freshly isolated blood samples. Whole blood samples were treated with PharmLyse (Pharmingen/B-D) to lyse the red blood cells, according to the manufacturer's recommendations. The lymphocytes were resuspended in PBS complemented with 2% FBS (P2F). Next, primary antibodies against feline CD4 (vpg39), CD8 (vpg9) [382] and a panT (f43) antibody [2] were added and incubated o/n at 4°C. After washing the cells, a PE-labeled goat antibody preparation against mouse IgG (DAKO) was added for 30 minutes at 4°C. The cells were washed once more, resuspended in PBS, and analysed by flow cytometry on a FACScan (BD). The data were analysed using CellQuest software.

Virus loads

Plasma virus loads were determined as described previously [147]. In brief, a real-time PCR was performed using the EZ-core kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The RT-PCR cycling program was initiated with a 2 min 50 °C uracyl amperase step, followed by a 30 min 60°C RT step, 5 min 95°C denaturation and 45 cycles of a 2 step PCR of 20 sec 95°C and 1 min 62°C. Data were collected during the annealing step (62°C). A diluted 19k1 virus stock was used in each run to obtain a standard-curve. 190 μl of feline plasma was spiked with a known Phocine Distemper Virus (PDV) stock that was used as an internal control to control for efficiency of RNA isolation. RNA was extracted from plasma with the Magna Pure LC Total Nucleic acid Isolation kit (Roche, Almere, The Netherlands).

Provirus loads or cell-associated virus loads were determined using an infectious centre test essentially as described [312]. Briefly, serially diluted PBMC samples obtained after challenge infection were co-cultured in 96-wells U-bottom plates (Greiner Bio-One, the Netherlands) with a Con-A and rhuIL-2 stimulated mixture of PBMC from two SPF cats in ten wells. Culture medium containing rhuIL-2 was added weekly to maintain the cultures. After four weeks the culture supernatants were analysed for the presence of FIV antigen by ELISA. The number of infected cells in the PBMC was

calculated from the results of the ELISA by assuming that one infected cell gave rise to FIV antigen production after co-cultivation with the stimulated PBMC, when one or more cultures tested in the ten wells were negative for FIV antigen.

IFN-γ ELIspot assay

Cryopreserved PBMC were tested for IFN-γ responses using an IFN-γ ELIspot assay (R&D Systems), essentially as described previsously [65]. Peptide pools containing overlapping 15-mer peptides spanning the entire proteins of FIV Rev and OrfA were used to stimulate PBMC samples. The 15-mer peptides had an overlap of 10 amino acids. The cells were incubated with 2mM Rev and OrfA peptide pools at a concentration of 5×10^5 cells/well. After 5 days of culture, 200 IU/ml rhIL-2 were added, and refreshed every two or three days for a period of 2-3 weeks. Next the cells were harvested and re-stimulated with peptide pools (2 μM of each peptide) in an ELIspot assay for 24 hours on MSIPS4W10 membrane plates (Millipore) coated with capture IFN-γ specific antibody. Control wells consisted of cells incubated with medium only or PMA/ionomycin at 50 ng/ml and 300 ng/ml, respectively. After 24 hrs, the cells were removed and the ELISPOT plates incubated overnight with a biotin-labelled IFN-γ specific antibody. Next, the plates were incubated with AP-labelled streptavidin (Dako) for one hour and developed with BCIP/NBT substrate

Statistical analyses

For statistical analysis of the data between two groups the Mann-Whitney test was used. *P* values of less than 0.05 were considered to be statistically significant.

Results

Vaccine-induced immune responses

Both the Rev and OrfA containing vaccines proved to be immunogenic. After the first immunization with these vaccines, antibodies to both proteins were detected by ELISA in the serum of vaccinated cats (Figures 2 and 3). Upon a booster vaccination the antibody levels increased considerably after which they were refractory to further boosting. In the cats vaccinated with the Env-ISCOM alone and in the mock vaccinated cats no antibodies to Rev and OrfA were detected.

In contrast, vaccination with the Env-ISCOM preparation failed to induce detectable Env-specific antibodies as measured by ELISA and VN assay (data not shown). Despite the lack of detectable Env specific antibody response, vaccination with Env-ISCOMs had primed for an anamnestic antibody response (see below) and hence had been immunogenic to a certain extent.

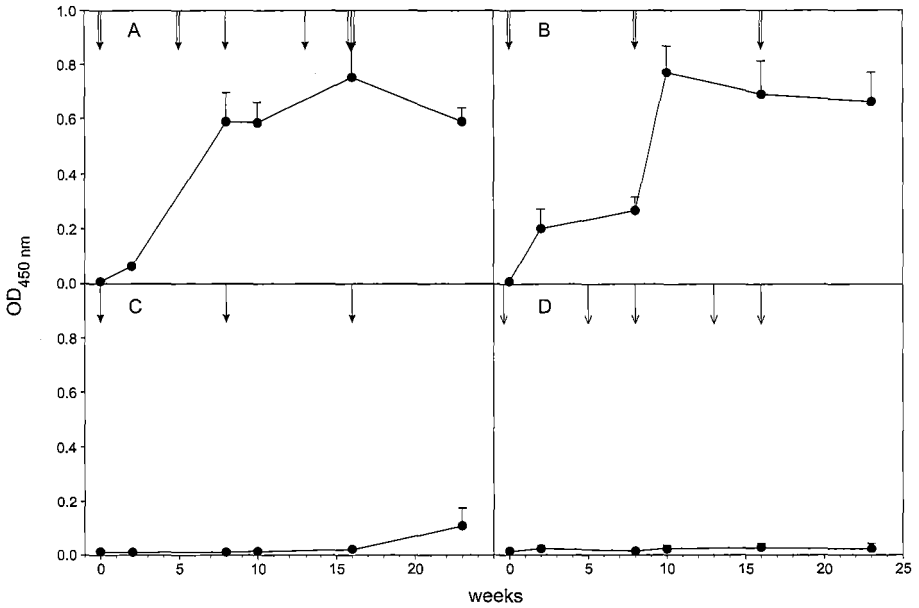

Figure 2. Rev-specific IgG responses in cats after vaccination as determined by ELISA.

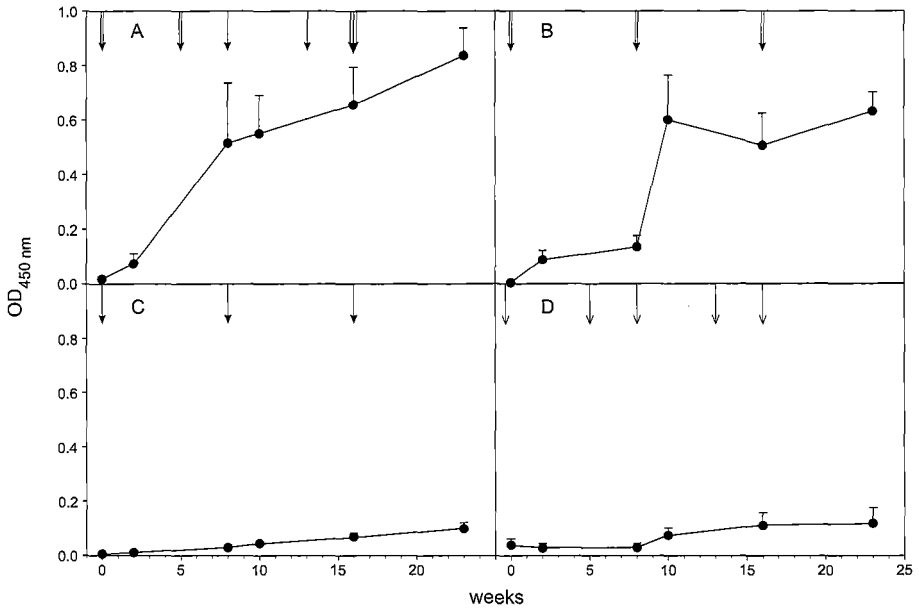

Figure 3. OrfA-specific IgG responses in cats after vaccination as determined by ELISA. The average reactivity with Rev (Figure 2) and OrfA (Figure 3) protein is shown for cats of group 1 (Rev/OrfA + Env, panel A), group 2 (Rev/OrfA, panel B), group 3 (Env only, panel C) and group 4 (mock, panel D). The error bars represent standard error of the mean. The arrows indicate the time points of the vaccinations: single arrow = Env vaccination, double arrow = Rev/OrfA vaccination, triple arrow = Rev/OrfA + Env vaccination, open-ended arrow = mock vaccination.

Immune responses post-challenge

After challenge infection with FIV 19k1, the antibody levels to Rev and OrfA did not further increase and remained stable (data not shown). As indicated above, vaccination with the Env-ISCOMs primed cats for a VN antibody response which was higher than the ones observed in the other groups ($P < 0.05$, Mann-Whitney test) (Figure 4). Furthermore, VN antibodies were detectable two to three weeks earlier than in the animals of the groups that did not receive the Env-ISCOM vaccine.

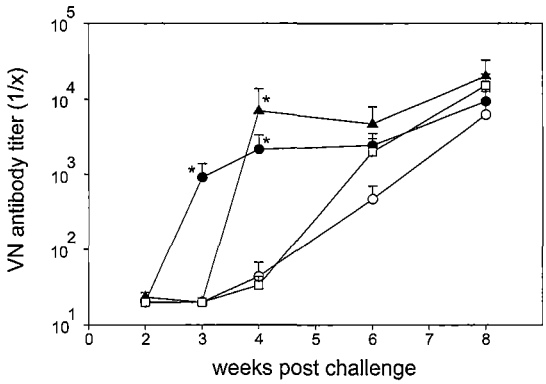

Figure 4. Virus neutralizing antibody responses after challenge infection with FIV. VN antibody titers were determined at indicated time points after FIV challenge infection in a CrFKcell-based virus neutralization assay [4]. The average antibody titers are shown for cats in group 1 (●), group 2 (○), group 3 (▲) and group 4 (□). The error bars indicate the standard error of the mean. Statistically significant differences are indicated by * ($P < 0.05$).

Outcome of challenge infection

In the groups of cats that received the Env-ISCOM vaccine, one animal became virus positive at one week after infection (1,700 copies/ml plasma). At two weeks post challenge, 9 out of 11 Env-ISCOM vaccinated cats were viremic, with virus loads ranging from 740 to 460,000 copies/ml (average 79,174, Figure 5A). At this time, three out of six cats were virus positive in the groups that received the Rev- and OrfA-ISCOMs without Env-ISCOM (830 – 3,000 copies/ml, average 1,055) and the mock vaccinated group (1,600 – 21,000 copies/ml, average 4,100). Although these kinetics may be suggestive of an enhancement of infection in cats vaccinated with Env-ISCOMs, this trend was not statistically significant. At later timepoints after infection, virus loads were similar for all groups of cats irrespective of the vaccine preparation used. A similar pattern was observed when the provirus load in PBMC during the first four weeks after challenge was analysed in an infectious centre test. Although the provirus loads in the Env-ISCOM vaccinated cats seem higher at two and three weeks post infection, no significant differences between the different groups of cats could be demonstrated (Figure 5B). The virus loads in the cats that were vaccinated with Rev-ISCOM and OrfA-ISCOM were very similar to those observed in the mock

vaccinated animals of group 4, indicating that also these animals were not protected from infection with FIV 19k1.

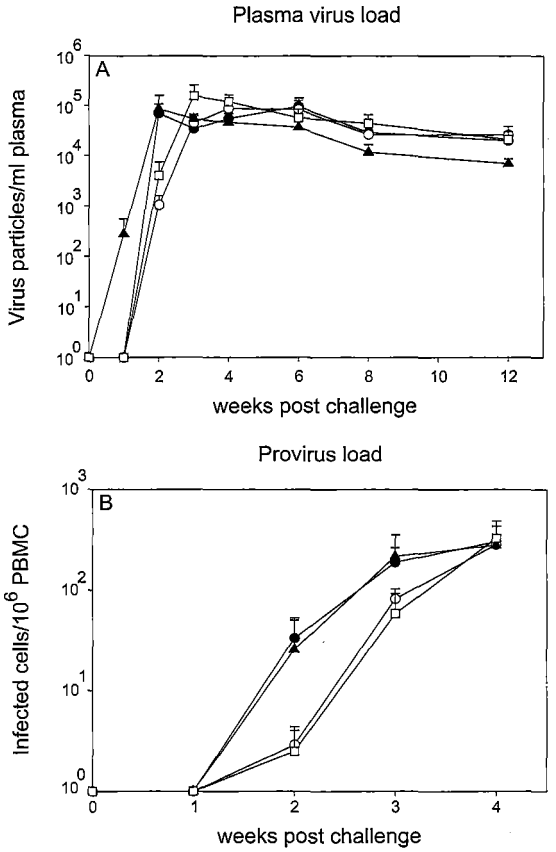

Figure 5. Virus loads after challenge infection with FIV. Virus loads were determined at the indicated time points postinfection either by Taqman analysis and presented as the average number of virus particles per ml plasma (A) or by an infectious center test and presented as the average infected number of cells per million PBMC (B), in cats of group 1 (●), group 2 (○), group 3 (▲) and group 4 (□).

Lymphocyte subset parameters

Analysis of the lymphocyte subsets after challenge infection showed a characteristic decrease of the CD4:CD8 lymphocyte ratio (Figure 6A). The cats of the Env-ISCOM vaccinated groups had a significantly lower ratio compared to the control and other vaccine groups at three and four weeks post challenge, respectively (P = 0.028 and 0.027, Mann-Whitney test). In all cases, this decrease was caused by a simultaneous drop in $CD4^+$ and a rise in $CD8^+$ lymphocyte numbers. Further analysis of the $CD8^+$ lymphocyte subset showed that this post challenge increase of $CD8^+$ lymphocytes was mainly caused by the appearance of a $CD8^{+lo}$ subpopulation (Figure 6B). In concurrence with the steeper decrease of the CD4:CD8 ratio, the

$CD8^{+lo}$ lymphocytes in the Env-ISCOM vaccinated cats of groups 1 and 3 constituted a significantly larger part of the $CD8^{+}$ lymphocyte population at four weeks after challenge ($P = 0.006$ and 0.045, respectively).

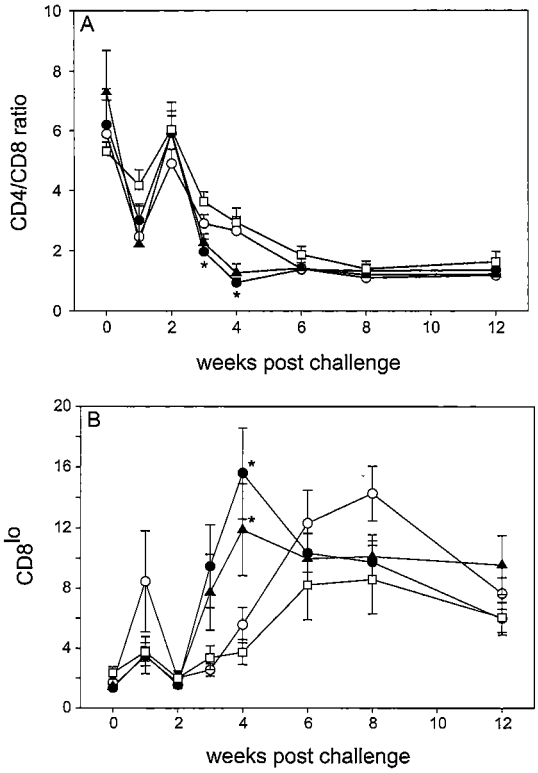

Figure 6. Analysis of T lymphocytes after challenge infection with FIV by flowcytometry. The average CD4:CD8 ratios (A) and frequencies of $CD8^{lo}$ T lymphocytes (B) in PBMC are shown at the indicated time points post-infection in cats of group 1 (●), group 2 (○), group 3 (▲) and group 4 (□) Statistically significant differences are indicated by * ($P < 0.05$).

Discussion

In the present study candidate FIV vaccines containing recombinant FIV Rev, OrfA and Envelope proteins were evaluated for their capacity to protect cats from infection with a homologous strain of FIV. None of the vaccine formulations induced protective immunity, despite inducing or priming antibody responses against the individual components of the vaccine. Virus replication was not prevented or delayed after vaccination with the respective vaccine preparations. The induction of antibodies against FIV Rev and OrfA was not sufficient to protect cats from infection. Using ISCOMs as a vaccine formulation, we also aimed at the induction of cellular

immune responses since it has been shown that the induction of cell mediated immunity is associated with protection in a number of lentivirus infection models [4,108,125,230,246,358]. We were unable to detect cellular immunity in the vaccinated cats by IFN-γ ELISPOT assays using PBMC and pools of overlapping peptides representing the complete open reading frames of FIV Rev and OrfA proteins. Only after infection with FIV, we were able to detect IFN-γ producing cells in 40% of the cats, independent of the vaccine that was used (data not shown), which indicates that it is possible to detect these responses by ELIspot using pools of overlapping peptides. It is possible that vaccination with the vaccine candidates induced cellular immune responses inefficiently. Since FIV infection induced Rev- and OrfA specific IFN-γ producing cells sporadically, it is possible that FIV Rev and OrfA proteins contain a small number of T-cell epitopes that can be presented by selected MHC class I molecules only. Indeed, using overlapping peptides and PBMC obtained from FIV-infected cats in an IFN-γ ELISPOT assay, it was shown that OrfA harboured a limited number of T cell epitopes compared to Gag, Env and reverse transcriptase (RT) proteins [65]. Also in an other study, the induction of virus specific cell-mediated immunity with Orf A-based vaccines was difficult and only transient responses were observed, shortly after vaccination [263]. It could by hypothesized that that cell-mediated responses occurred in lymphoid tissue and were not detected in PBMC. However it has been shown in the SIV-macaque model that the frequency of virus-specific T cells in the blood reflects the frequency in the spleen and were higher than in the lymph nodes [107]. Therefore, it is unlikely that we underestimated the virus-specific cell mediated immune responses by examining these in PBMC.

Vaccination of cats with Env-ISCOMs failed to induce a VN antibody response. A similar result was obtained previously with bacterially expressed env protein. In that study enhancement of infection was observed after vaccination with envelope protein that was expressed in an eukaryotic expression system, but not with bacterially expressed env protein [147,312]. In the present study with bacterially expressed env protein again no statistically significant enhancement of infection was observed, although there was a tendency for this. This may suggest that the enhancement of infection is not attributable to glycosylation of the protein [147,312].

In the Env-ISCOM vaccinated cats a lower CD4:CD8 ratio at three and four weeks post infection was observed than in the other cats (Figure 6A). This reduction could mainly be attributed to a sharp rise in $CD8^{+lo}$ lymphocytes in these two groups (Figure 6B). This subpopulation of $CD8^{+}$ lymphocytes has been described previously during the course of FIV infection and most likely are activated T cells since their CD62L expression is down-regulated. They exert antiviral activity in *in vitro* infection assays [102]. This population of $CD8^{+lo}$ T lympocytes can make up to 80% of the total $CD8^{+}$ T cell population and contains all FIV specific TNF-α secreting T cells [240]. We speculate that

the tendency towards an accelerated viremia observed in the Env-ISCOM vaccinated cats contributed to the rapid induction of this $CD8^{+lo}$ T lymphocyte population.

Collectively, the data presented here indicate that the use of FIV Rev and OrfA proteins either alone or in combination with fragments of bacterially expressed envelope protein did not confer protection against challenge infection with FIV in cats. The use of the Envelope protein in vaccine preparations influenced the outcome of infection in a negative way. Although the use of accessory proteins as vaccine candidates has been succesful in some animal models for lentiviral infections, their use in combination with ISCOMs as an adjuvant was not succesful in the FIV/cat model. Clearly more research is required to understand how protective immunity can be induced best using these proteins. It is likely that other vehicles or vectors will be needed or combinations with other targets for cell mediated immunity for the induction of protective immunity against FIV in particular and lentiviruses in general.

5

Evaluation of vaccination strategies against infection with feline immunodeficiency virus (FIV) based on recombinant viral vectors expressing FIV Rev and OrfA

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Abstract

In recent years it has become clear that cell-mediated immunity is playing a role in the control of lentivirus infections. In particular, cytotoxic T lymphocyte responses have been associated with improved outcome of infection, especially those directed against the regulatory proteins like Rev and Tat, which are expressed early after infection. Therefore, there is considerable interest in lentiviral vaccine candidates that can induce these types of immune responses. In the present study, we describe the construction and characterization of expression vectors based on recombinant Semliki Forest virus system and modified vaccinia virus Ankara for the expression of feline immunodeficiency virus (FIV) accessory proteins Rev and OrfA. These recombinant viral vectors were used to immunize cats using a prime-boost regimen and the protective efficacy of this vaccination strategy was assessed after challenge infection of immunized cats with FIV.

Introduction

Since its discovery two decades ago, feline immunodeficiency virus (FIV) infection of cats has been used extensively as a small animal model to study lentivirus pathogenesis and for the development of candidate lentivirus vaccines [76,84,356,383]. Although various vaccine strategies have been evaluated, the immune correlates of protection are still largely unclear. Early attempts to develop lentiviral vaccines focussed on the induction of virus neutralising antibodies specific for the envelope glycoprotein. However, more recently it has become clear that virus-specific cell-mediated immunity, in particular cytotoxic T lymphocytes (CTL), contribute to control of lentivirus infection. In human immunodeficiency virus-1 (HIV-1) infected humans strong anti-HIV-1 CTL immunity correlated with reduced virus loads [230]. CTL responses against the regulatory proteins Rev and Tat in addition to Gag and RT were detected in long-term asymptomatic HIV-1 infected individuals, but not in patients rapidly progressing to acquired immunodeficiency syndrome (AIDS) [4,125,246,358]. Also in macaques experimentally infected with simian immunodeficiency virus (SIV), control of viral replication correlated with Rev and Tat specific CTL responses [108]. Therefore, the induction of CTL responses against the early regulatory proteins Rev and Tat could be important targets for candidate lentivirus vaccines. Indeed, Rev- and Tat-specific CTL induced with recombinant viral vectors more efficiently controlled virus replication in SIV-challenged macaques than Gag- and Pol-specific CTL [238,330].

For the induction of CTL responses special antigen delivery systems are required to direct proteins of interest into the endogenous route of antigen processing and major histocompatibility complex (MHC) class I restricted presentation. Recombinant viral vectors fulfill this requirement and are able to induce CTL responses against the protein of interest that is expressed. Examples include recombinant Semliki Forest Virus (SFV) [187] and replication deficient poxvirus vectors, such as modified vaccinia virus Ankara (MVA) [207], both of which have been used successfully to induce cellular immunity against various viral infections [20,47,329,335,392,399]. Strong cell-mediated immune responses have been achieved in the SIV-macaque model with prime-boost vaccination regimens using SFV and MVA vectors [226,238,330,367].

In the present study prime-boost vaccination strategies based on a SFV expression system and MVA were evaluated for their capacity to induce protective immune responses against the FIV accessory proteins Rev and OrfA. To this end, the respective expression vectors were constructed, characterized and subsequently used to immunize cats. Subsequently, immunized cats were infected with a homologous strain of FIV to assess the protective potential of this vaccination strategy.

Materials and Methods

Cells and viruses

Feline peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples using Lymphoprep (Axis-Shield, Norway), aliquoted and cryopreserved. After thawing, PBMC were allowed to recover overnight in RPMI (Cambrex Bioscience, Belgium) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 2×10^{-5} M ß-mercaptoethanol and 10% foetal bovine serum (FBS) (Greiner Bio-One, the Netherlands; R10F). Next, the cells were washed and subsequently cultured in R10F containing concanavalin A (4 μg/ml) (Sigma-Aldrich) and recombinant human interleukin-2 (rhuIL-2) (200 IU/ml) (Red Swan pharma logistics, the Netherlands) and used to determine provirus loads in an infectious centre test (see below).

Primary chicken embryo fibroblasts (CEF), baby hamster kidney BHK-21 cells and rabbit kidney RK-13 cells were grown in minimal essential medium (MEM, Cambrex Bioscience, Belgium) supplemented with 10% FCS.

Molecularly cloned FIV 19k1 was used as the challenge virus [309]. This virus was originally cloned from bone marrow DNA of a naturally infected (FIV AM19) cat and an in vitro grown virus stock was subsequently titrated in vivo [312]. Its sequence identity to the recombinant proteins used in the vaccine preparations is 100%.

Generation of recombinant MVA

The rev and orfA open reading frames of FIV 19k1 [309] were amplified by PCR using Pfu polymerase (Stratagene), pThioHisV5 expressions plasmids [147] as a template and cloned into the MVA transfer plasmid pIIIdhrP7.5, essentially as described previously [322]. The identity of the inserts was confirmed by restriction enzyme analysis and sequencing. BHK-21 cells were infected with MVA using a multiplicity of infection of 0.02 and then transfected with pIIIdhrP7.5-Rev or pIIIdhrP7.5-OrfA using Lipofectin (Invitrogen). Two days post infection, the cells were sonicated after three rounds of freeze-thawing and used to infect RK-13 cells in ten-fold serial dilutions. After three days, individual foci were collected and recombinant viruses were grown in RK-13 cells. After four passages, the clonal recombinant MVA were passaged three times in BHK-21 cells, which facilitates deletion of the K1L gene from the MVA genome through homologous recombination. The identity and the selection of the recombinant MVA were confirmed by PCR. To this end, DNA was isolated from infected cell cultures and used as template in the PCR using oligonucleotides targeting the flanking regions of deletion III. Insertion of FIV rev and orfA was confirmed using oligonucleotides used for the amplification of these genes. In addition, K1L specific primers were used to confirm deletion of the vaccinia virus K1L gene. Ultimately,

virus stocks were grown in primary chicken embryo fibroblasts and infectious virus titers were determined by vaccinia virus specific immunostaining. The identity of the inserts was confirmed by sequence analysis.

Generation of SFV DNA constructs

The rev and orfA open reading frames of FIV 19k1 [309] were amplified by PCR with Pfu polymerase (Stratagene), using pThioHisV5 expressions plasmids [147] as a template and subsequently cloned into the pSHAMEb2 plasmid. The identity of the inserts was confirmed by restriction enzyme analysis and sequencing.

Characterisation of vacccines

To confirm that rev and orfA mRNA is transcribed after infection of BHK-21 cells with recombinant MVA or after transfection with recombinant pSHAMEb2 plasmids, RNA was extracted from these cells twenty-four hours post-infection with rMVA-Rev or rMVA-OrfA or post-transfection of pSHAMEb2-rev or pSHAMEb2-orfA using the High Pure RNA purification kit (Roche) and an additional DNase treatment. The RNA was subsequently used in RT-PCR using primers originally used for the amplification of the rev and orfA genes. RT-PCR was carried out in the presence and absence of RT enzyme (Superscript III; Invitrogen) to exclude amplification on basis of template DNA.

Protein expression of Rev and OrfA was tested by SDS-PAGE and Western blot of lysates of recombinant MVA infected BHK-21 cells. For the detection of the FIV proteins sera were used from rabbits immunized with Rev and OrfA peptides (Eurogentec) and a horseradish peroxidase (HRPO) labeled swine anti rabbit IgG-antibody preparation. The blots were developed with a chemiluminescence substrate (ECL kit, Amersham).

Cats, vaccination and challenge infection

SPF cats were purchased from Harlan (Horst, the Netherlands). Eighteen animals were randomly assigned to three groups of six animals. The cats in group 1 were vaccinated twice with 150 μg DNA of the SFV constructs followed by two immunizations with 10^8 PFU of rMVA-Rev and rMVA-OrfA. All immunizations were given by the intramuscular route with four-week time intervals. The cats in group 2 were immunized with the OrfA constructs only and the cats in group 3 were mock vaccinated with wildtype MVA and the pSHAMEb2 plasmid.

Four weeks after the last vaccination the cats were infected with 20 CID_{50} of FIV molecular clone 19k1 by intramuscular injection. Plasma and PBMC were collected at

regular time intervals before and after vaccination and on weeks 1, 2, 3, 4, 6, 7, 10 and 12 after challenge infection.

ELISA for the detection of Rev and OrfA specific antibodies

To detect Rev and OrfA specific antibody responses, ELISA plates (Greiner) were coated with maltose-binding protein (MBP), MBP-Rev or MBP-OrfA fusion proteins at 100 ng per well in PBS. Subsequently, 100 µl volumes of cat plasma were added at a dilution of 1:800 (MBP-Rev) or 1:400 (MBP-OrfA), which were found to give optimal signal to noise ratios. Next, the plates were incubated with a mouse anti cat IgG antibody preparation (Serotec), followed by HRPO-labeled rabbit anti mouse IgG antibodies. All incubations were carried out for one hour at 37°C and after each incubation step the plates were washed with PBS containing 0.05% Tween-20. Subsequently, the plates were developed using 3,3',5,5' tetramethylbenzidine substrate (Meddens). After stopping the colour reaction with 1M H_2SO_4, OD_{450} values were determined by subtracting the OD_{450} values obtained with MBP from those obtained with MBP-Rev and MBP-OrfA.

Vaccinia virus-specific antibody response

Vaccine-induced MVA-specific antibody responses were determined essentially as described previously [329]. RK-13 cells were infected with wild-type vaccinia virus at a multiplicity of infection of 10. Seven hours after infection, the cells were trypsinized and used as target cells in a fluorescence-activated cell sorter (FACS)-measured immunofluorescence assay. The cells (1×10^5 to 3×10^5 cells/100 µl) were incubated with plasma samples diluted 1:100 in PBS supplemented with FBS. After a one-hour incubation on ice, cells were washed and subsequently incubated with a FITC-labelled monoclonal antibody directed to cat IgG.

After another hour on ice, cells were washed twice with PBS and fixed with 1% paraformaldehyde for 20 min on ice. Fluorescence signals were measured by using a FACScan (Becton Dickinson). Fluorescence intensity was quantified by determining the geometric mean of the fluorescence signal.

IFN-γ ELIspot assay

Cryopreserved PBMC were tested for IFN-γ responses using an IFN-γ ELIspot assay (R&D Systems), essentially as described previsously [65]. Peptide pools containing overlapping 15-mer peptides spanning the entire proteins of FIV Rev and OrfA were used to stimulate PBMC samples. The 15-mer peptides had an overlap of 10 amino acids. The cells were incubated with 2mM Rev and OrfA peptide pools at

a concentration of 5×10^5 cells/well. After 5 days of culture, 200 IU/ml rhIL-2 were added, and refreshed every two or three days for a period of 2-3 weeks. Next the cells were harvested and re-stimulated with peptide pools (2 μM of each peptide) in an ELIspot assay for 24 hours on MSIPS4W10 membrane plates (Millipore) coated with capture IFN-γ specific antibody. Control wells consisted of cells incubated with medium only or PMA/ionomycin at 50 ng/ml and 300 ng/ml, respectively. After 24 hrs, the cells were removed and the ELIspot plates incubated overnight with a biotin-labelled IFN-γ specific antibody. Next, the plates were incubated with AP-labelled streptavidin (Dako) for one hour and developed with BCIP/NBT substrate.

FIV loads post infection

Plasma virus loads were determined as described previously [147,312]. In brief, a real-time PCR was performed using the EZ-core kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The RT-PCR cycling program was initiated with a 2 min 50°C uracyl amperase step, followed by a 30 min 60°C RT step, 5 min 95°C denaturation and 45 cycles of a 2 step PCR of 20 sec 95°C and 1 min 62°C. Data were collected during the annealing step (62°C). A diluted 19k1 virus stock was used in each run to obtain a standard-curve. 190 μl of feline plasma was spiked with a known Phocine Distemper Virus (PDV) stock that was used as an internal control to control for efficiency of RNA isolation. RNA was extracted from plasma with the Magna Pure LC Total Nucleic acid Isolation kit (Roche, Almere, The Netherlands).

Provirus loads or cell-associated virus loads were determined using an infectious centre test essentially as described [147,312]. Briefly, serially diluted PBMC samples obtained after challenge infection were co-cultured in 96-wells U-bottom plates (Greiner Bio-One, the Netherlands) with a Con-A and rhuIL-2 stimulated mixture of PBMC from two SPF cats in ten wells. Culture medium containing rhuIL-2 was added weekly to maintain the cultures. After four weeks the culture supernatants were analysed for the presence of FIV antigen by ELISA. The number of infected cells in the PBMC was calculated from the results of the ELISA by assuming that one infected cell gave rise to FIV antigen production after co-cultivation with the stimulated PBMC, when one or more cultures tested in the ten wells were negative for FIV antigen.

Results

Characterisation of the vaccine preparations

Cell lysates of rMVA-Rev and rMVA-OrfA infected BHK-21 cells were tested for the presence of Rev and OrfA by SDS-PAGE and Western blot analysis. As shown

in Figure 1, expression of Rev by rMVA-Rev was detected whilst OrfA expression was not. However, for both Rev and OrfA transcription of mRNA was detected in rMVA infected cells (Figure 2). Also in cells transfected with the SFV constructs, Rev and OrfA transcription was detected (Figure 2), although the presence of Rev and OrfA protein in these cells could not be detected by Western blot analysis (data not shown).

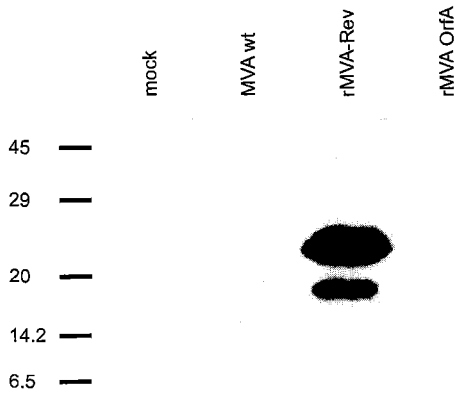

Figure 1. Rev expression by rMVA-Rev. Wildtype MVA (MVAwt), rMVA-OrfA, rMVA-Rev and mock infected BHK-21 cells were harvested twenty-four hours after infection and lysed with SDS-PAGE loading buffer. Specific protein was visualized by SDS-PAGE and Western blot analysis.

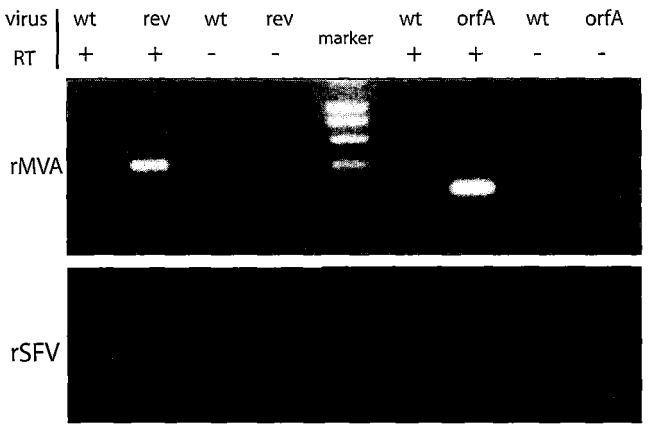

Figure 2. Transcription of Rev and OrfA mRNA by rMVA (upper panel) and rSFV (lower panel) constructs. RNA was isolated from infected (rMVA) or transfected (rSFV) BHK-21 cells and used as template in RT-PCR. PCR was performed using rev or orfA specific primers in absence (-) or presence (+) of reverse transcriptase (RT) enzyme, to exclude possible contamination with DNA.

Vaccine-induced immune responses

In none of the cats antibodies to either Rev or OrfA were detected at any time point after vaccination. However, after immunization with the rMVA vaccine preparations, antibodies to vaccinia virus proteins were detected (Figure 3), indicating that rMVA vaccines were immunogenic and that rMVA specific antibody titers increased after the booster vaccination.

Cellular immunity against Rev and OrfA was measured using an ELIspot assay for the detection of IFN-γ secreting lymphocytes after in vitro restimulation of PBMC with Rev and OrfA peptide pools. However, no evidence of vaccine-induced Rev- or OrfA-specific cellular immune responses was found in the vaccinees.

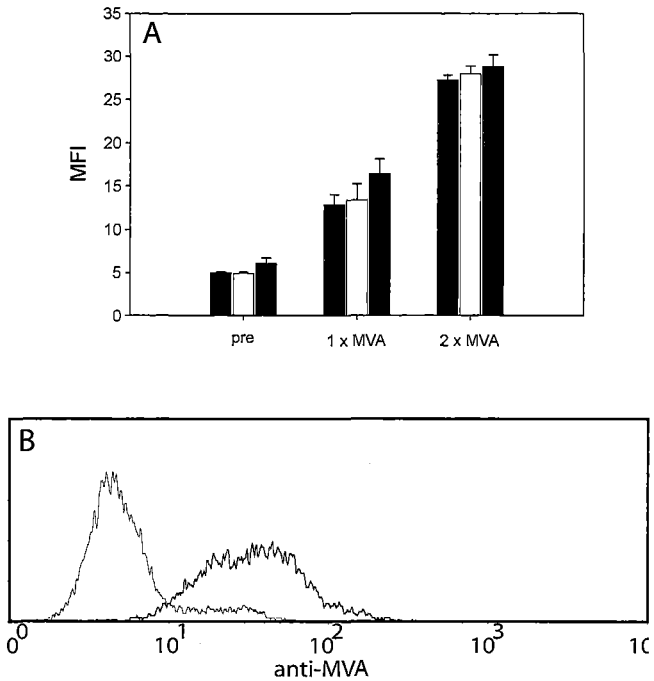

Figure 3. MVA specific antibody responses. (A) Vaccine-induced antibody responses against the MVA vector were detected in plasma samples taken before vaccination (pre) and four weeks after the first (1xMVA) and second vaccination (2xMVA) in a flow cytometry-based assay using vaccinia virus infected RK-13 cells. Antibody reactivity is expressed as the geometric mean of the fluorescence. (B) A representative histogram in which the thin curve is obtained using pre-vaccination plasma and the bold curve with plasma obtained after the second vaccination.

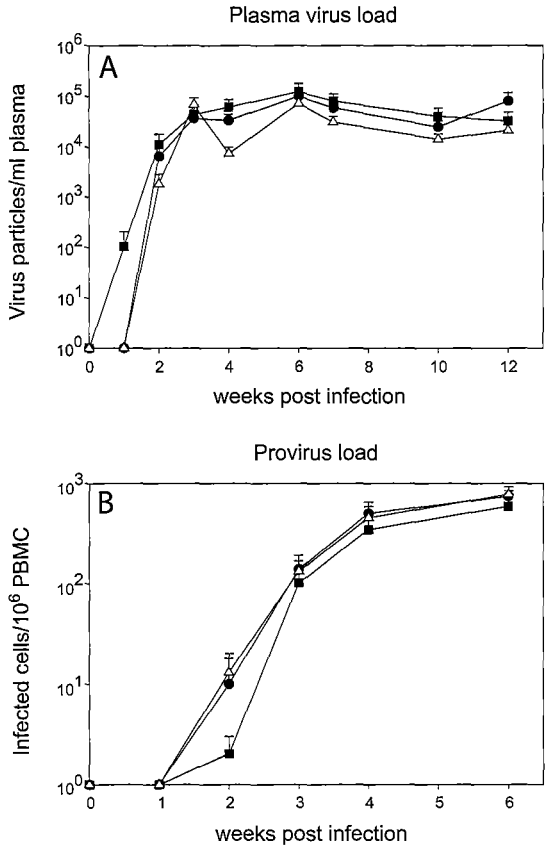

Figure 4. Plasma (A) and provirus (B) loads. Virus loads were determined either by Taqman analysis and expressed as the average number of virus particles per ml plasma (A) or by an infectious centre test and expressed as the average infected number of cells per million PBMC (B). Group 1 (■), Group 2 (●), Group 3 (▲).

Outcome of challenge infection

Four weeks after the last vaccination, the cats were infected with 20 CID_{50} of FIV strain 19k1. As shown in Figure 4, the average plasma viral loads and proviral loads measured in Rev and OrfA vaccinated cats of group 1 and group 2 did not significantly differ from those measured in the control cats of group 3, indicating that protective immunity was not induced. Eventually all cats became viremic (Table 1), although the number of Rev and OrfA vaccinated cats of group 1 positively tested for the presence of virus in the plasma was somewhat delayed compared to the control group 3 ($P = 0.04$, Logrank test). At two weeks post challenge, two cats of group 1 tested positive, whereas in groups 2 and 3 this number was 4 and 6, respectively. At three weeks post challenge, only one cat of group 2, vaccinated with OrfA alone, tested negative for virus in the plasma. By week four all cats tested positive (Table

1). Statistically significant differences between groups were not observed for the presence of provirus in these cats (data not shown).

In addition, no differences were observed in the post challenge fluctuations in the CD4:CD8 ratios between vaccinated and control cats (data not shown).

Table 1. Number of individual cats test positive for the presence of virus in plasma

	Group		
	1	2	3
Weeks p.i.	Rev/OrfA	OrfA	Control
0	0/6*	0/6	0/6
1	1/6	1/6	2/6
2	2/6	4/6	6/6
3	6/6	5/6	6/6
4	6/6	6/6	6/6

*Number of FIV positive cats per group, as determined using Taqman real-time PCR on plasma samples collected at indicated time points post infection.

Discussion

In the present study, SFV and MVA-based vector vaccines were prepared for the delivery of the FIV regulatory proteins Rev and OrfA. Subsequently these vaccine candidates were evaluated in cats for their capacity to induce protective immunity against FIV. The two viral vectors were used in a prime-boost regimen in order to induce optimal immune responses. However, the induction of humoral and cell-mediated immunity could not be detected and the vaccination strategy failed to confer protection against infection with FIV. These results are disappointing and there may be several reasons that might explain this vaccine failure. Although for both vector systems it was demonstrated that rev and orfA mRNA transcription took place in BHK-21 cells, only the production of Rev protein was convincingly demonstrated after infection with rMVA-Rev. This might refect low expression of these proteins under these circumstances. Also in FIV-infected cats the expression of these proteins is extremely low, especially for OrfA and it has been suggested that OrfA is a very unstable protein, rapidly degraded through the ubiquitin-mediated pathway for proteolysis [46]. Thus, it is unclear whether the antigen load in vivo was sufficiently high for the induction of an immune response. This lack of immunogenicity was not related to problems with the vectors since antibodies directed to MVA were readily detected after administration of MVA in all these groups. The intracellular localization, in combination with low expression levels may have further prevented recognition of Rev and OrfA by specific B-cells and thus prevented a B cell response. The cytosolic localization of protein antigens is a prerequisite for the efficient induction

of virus specific CTL responses. The failure to detect any Rev or OrfA-specific cellular immune response using an IFN-γ ELIspot assay and pools of overlapping peptides especially is disappointing since the vaccination approach specifically aimed at the induction of these responses. Since Rev and OrfA are relatively small proteins the number of epitopes that can be recognized by feline T lymphocytes may have been too limited to induce appreciable T cell responses. In addition, MHC restriction of T cell responses may have contributed to the apparent lack of T cell responses, since in experimentally infected (outbred) cats we have only been able to detect Rev and OrfA specific T cell responses in about 40% of the cats (data not shown). These findings are in concordance with other studies which demonstrated that after OrfA DNA vaccination T cell responses were detected only transiently early after vaccination which suggested that these were momentary and refractory to boosting [263]. Also after vaccination with another vaccine candidate that was based on recombinant OrfA and Rev proteins and the use of immune stimulating complexes (ISCOMs), no CTL responses were observed. In addition, there was no evidence that vaccination primed CTL responses after subsequent challenge infection [148]. Thus, the outcome of vaccination with the OrfA and Rev expressing vector vaccines tested in the present study resembled that with the OrfA/Rev-ISCOM vaccines.

Although the cats in group 1, vaccinated with the Rev and OrfA constructs, became viremic slightly later than the cats in the other two groups suggests that the inclusion of Rev in the vaccine preparation contributed to this transient protective effect. However, ultimately none of the vaccinated cats proved to be protected.

With vaccination strategies aiming at the induction of T cell immunity against the regulatory proteins Rev and Tat protective effects were achieved in the SIV-macaque model in some studies [40,238,330]. This is in agreement with the strong correlation observed in HIV-1 infected humans between CTL responses against these proteins and reduced viral loads [4,125,246,358]. Apparently these positive results could not be reproduced in the FIV-cat model and as such this model may be poorly predictive for the induction of protective CTL against lentiviral regulatory proteins in primates. On the other hand, also in the SIV-macaque model vaccine failure has been reported with vaccines inducing Tat-specific CTL responses [5,6,184,214,314]. Furthermore, a clinical trial which evaluated adenoviral vectors for their capacity to induce CTL responses to Gag, Pol and Nef and protective immunity to HIV-1 in subjects at high risk for exposure was halted recently. Despite the induction of CTL responses to the respective proteins, this vaccine was not effective in preventing infection [44,73,224]

Collectively, the data presented in the present study indicate that the use of the SFV and rMVA vector based vaccines for the expression of FIV Rev and OrfA is not sufficient for the induction of protective immunity in cats against FIV. The conflicting results obtained in the SIV-macaque model and disappointing trials with a vaccine

inducing CTL responses to Gag, Pol and Nef indicate that the development of a vaccine that aims at the induction of CTL responses is not straight forward and more research is required to assess the minimal requirements for CTL-based protective immunity against lentiviruses.

6

INTRAHOST EVOLUTION OF ENVELOPE GLYCOPROTEIN AND ORFA SEQUENCES AFTER EXPERIMENTAL INFECTION OF CATS WITH A MOLECULAR CLONE AND A BIOLOGICAL ISOLATE OF FELINE IMMUNODEFICIENCY VIRUS

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Abstract

Feline immunodeficiency virus (FIV) is a member of the genus *Lentivirus* and causes AIDS-like disease in its natural host, the cat. Like other lentiviruses, FIV displays a high degree of nucleotide sequence variability that is reflected in both the geographic distribution of the viruses and the different cat species that are infected. Although a lot of data on sequence variation at the population level is available, relatively little is known about the intrahost variation of FIV sequences. In the present study, cats were infected with either a biological isolate of FIV or a molecular clone that was derived from the same isolate, AM19. After infection, the cats were monitored for up to three years and at various time points sequences were obtained of virus circulating in the plasma. Regions of the *env* gene and the *orfA* gene were amplified, cloned and their nucleotide sequence analyzed. Furthermore, the extent of sequence variation in the original inocula was also determined. It was found that FIV is displaying relative little sequence variation during infection of its host, both in the *env* and the *orfA* gene, especially after infection with molecular clone 19k1. Although the extent of variation was higher after infection with biological isolate AM19, a large portion of these variant sequences was already present in the inoculum.

Introduction

Feline immunodeficiency virus (FIV), first discovered in 1986 [251], is a member of the *Retroviridae* family, genus lentivirus. FIV infection of cats shares multiple biological and clinical characteristics with HIV-1 infection of man. Because of these similarities in immunopathogenesis, FIV infection of cats is considered a valuable animal model for AIDS [18,78,383]. During its replication cycle, the viral genome integrates into the host's chromosomal DNA. From the infected cells, virus is produced also during the asymptomatic stage of the infection, although to a lower extent than during the initial phase. For the integration process, reverse transcription of the single stranded viral RNA genome into a DNA intermediate molecule is required. This step is carried out by a virally encoded reverse transcriptase (RT) enzyme, an RNA-dependent DNA polymerase, present in mature virions of all members of the *Retroviridae*. Since this enzyme lacks a proofreading mechanism that corrects for erroneously incorporated nucleotides, this step in the viral replication cycle is at the basis of extensive sequence variation, a typical feature of lentiviruses that includes base substitution, addition, and deletion [288]. Most research into this phenomenon focuses on viral genetic variation on the population level, describing the demographics of lentivirus infections and phylogenetic analyses on the variation of viral sequences within populations [7,36,171,278,303].

Also for FIV, studies on sequence variation mainly describe geographic, demographic and species specific differences at the population level [42,232,259,268,316]. On the basis of envelope glycoprotein variable regions 3 to 5, FIV has been classified into five subtypes [9,159,250]. In contrast, information on intrahost sequence variation is sparse and limited [116,145,151,218]. In most cases, intrahost sequence variation has been assessed based on sequence analyses of PCR amplicons obtained using DNA isolated from the PBMC of infected cats as a template and therefore reflect the most abundantly present proviral genomes rather than the repertoire of virus sequences in circulation.

We wished to assess the intrahost variation of FIV after infection of its natural host. To this end, cats were inoculated with either a biological FIV isolate (FIV AM19) or a molecular clone obtained from the same cat (FIV 19k1) [309,312]. To relate the variation observed in vivo with that of the input virus, the extent of sequence variation in the virus inoculum was determined as well. At several time points post infection, regions of the *env* gene and the complete coding sequence of the *orfA* gene were amplified by RT-PCR using RNA extracted from plasma as template and cloned. Subsequently, the nucleotide sequence of up to twelve clones per amplicon was determined. The Env glycoprotein was chosen since it constitutes a major target of humoral immune responses [243]. All variable regions (V3 to V9) located in the surface (SU) and transmembrane (TM) subunits of the mature Env glycoprotein were analyzed for

sequence variation. In contrast to Env, OrfA, a small transactivator protein involved in the regulation of FIV replication, is expressed intracellularly only [46,56,106] and is a target for cell mediated immune responses [65]. It was found that in this model for lentivirus infection in a natural host, the intrahost variation of FIV sequences was limited even after three years post infection.

Materials & Methods

Viruses and animals

Four cats, five months of age, were infected intraperitoneally (i.p.) with $10^{3.5}$ 50% cat infectious doses of FIV; two cats (11 and 12) were inoculated with biological isolate FIV AM19 and two cats (13 and 14) with molecular clone FIV 19k1, originally isolated from the same animal from which the biological isolate was obtained [309,312]. After challenge, peripheral mononuclear blood cells (PBMC) and plasma samples were obtained from blood regularly for a period of up to 143 weeks and stored at –135°C (PBMC) or –70°C (plasma).

RNA isolation, RT-PCR and molecular cloning

Total RNA was isolated from plasma collected in heparin tubes (1 ml input, elution volume: 50μl) using a RNA isolation kit (Macherey-Nagel, Düren, Germany) or the Magnapure RNA extraction (Roche Diagnostics, Almere, the Netherlands). Subsequently, 5 – 10 μl was used as template in a RT reaction using Superscript III (Invitrogen, Breda, the Netherlands) and random hexadeoxynucleotide primers (Promega Benelux BV, Leiden, the Netherlands). Reactions were carried out according to the manufacturer's protocol. The obtained cDNA was used as a template in the subsequent PCR. Primers used were 5'-CAGGAGGAAAAATGTTGTA-3' and 5'-AATAGCTGCTCTTTTTCTTC-3' for the amplification of the envelope glycoprotein regions V3 through V6, 5'-AAGGACGGGGGCTACTGCTAT-3' and 5'-TGCCACATTGCCTACCATTTCTC-3' for the amplification of the envelope glycoprotein regions V7 through V9, and 5'-AGCTGGAGTCTGGCCCTTCTTTCA-3' and 5'-GGATTTAGTGCCCCTTCTTCA-3' for the *orfA* open reading frame. Phusion DNA polymerase enzyme (Finnzymes, Espoo, Finland), which has proofreading capacities, was used in the PCR. After denaturation at 98°C, 34 (*orfA*) or 39 (*env* V3-V6, V7-V9) cycles were performed of denaturation, annealing at 51°C (V3-V6 and *orfA*) or 60°C (V7-V9) and extension for 60 (V3-V6 and V7-V9) or 15 (*orfA*) seconds at 72°C. After the last cycle reactions were continued for 10 minutes at 72°C.

After RT-PCR, the obtained fragments were separated on a 1% agarose gel and isolated using the Qiaprep Gel Extraction kit (Qiagen Benelux BV, Venlo, the Netherlands). After a 10 minute reaction with Taq polymerase (Promega Benelux BV, Leiden, the Netherlands) and dNTP to add an adenosine deoxynucleotide to their 5' ends, the purified fragments were cloned into pCR4 using the TOPO TA cloning kit according to the manufacturer's protocol (Invitrogen, Breda, the Netherlands).

Sequencing and analysis

Sequences of up to 12 individual clones of each construct were obtained using M13 forward and reverse primers. Sequences were obtained on an ABI3100 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and analyzed using the Lasergene software package (DNASTAR Inc., Madison, WI, USA). DNA sequence alignments and analyses were carried out using BioEdit software. For the envelope glycoprotein sequence analyses, fragments of 894 and 618 nucleotides of the V3-V6 and V7-V9 regions were used, respectively. For *orfA* sequence analyses, the complete 237 nucleotide open reading frame was used.

Plasma virus load and virus reisolation

Plasma virus load was determined by a real-time PCR assay (Taqman), using an ABIprism 7700 Sequence Detection System, as described previously [147].

Virus reisolation from plasma was performed by inoculating 200 µl of plasma with 2×10^6 target cells (a mix of PBMC cultures obtained from two different FIV negative cats) in 2 ml RPMI (Cambrex Bioscience, Belgium) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), ß-mercaptoethanol ($2 \cdot 10^{-5}$ M), 10% fetal bovine serum (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) and recombinant human interleukin-2 (200 IU/ml) (Red Swan Pharma Logistics, Utrecht, the Netherlands) in a 24-wells culture plate. Every three to four days, 0.5 ml of medium was harvested and stored at –20°C for analysis of RT-levels. RT activity was determined with the Lenti-RT activity kit (Cavidi Tech, Uppsala, Sweden).

Results

Sequence variation in virus stocks

Molecular clone 19k1

Analyses of the viral sequences present in the virus stock of molecular clone 19k1 showed that 80% of the clones tested were identical to the published 19k1 Env amino acid sequence (GenBank accession no. **M73964**) for variable regions V3 through

V6 (table 1a, upper panel). Two clones contained a single non-synonymous point mutation, one of which was present in V3. Therefore, the 19k1 consensus sequence constitutes the majority of the viruses present in the 19k1 virus stock. As shown in tables 1b and 2 (upper panels), the 19k1 virus stock exhibited no sequence variation in either envelope regions V7 through V9 or the OrfA protein.

Table 1a. Sequence variation in SU regions V3-V6 in 19k1 infected cats

				V3							
source	time	n	aa# >	319	324	348	406	445	451	528	582
19k1		8		L	L	D	F	C	K	I	Q
		1		-	-	-	-	-	-	S	-
		1		-	-	-	L	-	-	-	-
cat 13	8	8		-	-	-	-	-	-	-	-
		1		-	-	-	-	-	E	-	-
	44	2		-	-	-	-	-	-	-	-
		1		-	-	-	-	*	-	-	-
		1		-	-	-	-	-	-	-	P
		1		X	-	-	-	-	-	-	-
		1		X	-	Y	-	-	-	-	-
		1		-	V	-	-	-	-	-	-
cat 14	32	8		-	-	-	-	-	-	-	-
	44	8		-	-	-	-	-	-	-	-
	143	4		-	-	-	-	-	-	-	-

X = one nucleotide deletion, * = premature stopcodon, aa# = amino acid no. All tables are compiled from sequence alignment data using the following format: in the first line amino acid positions in the open reading frames of either Env or OrfA are given that deviate from the consensus sequence. The upper panel shows amino acid variants found in the virus stocks (either molecular clone 19k1 or biological isolate AM19). The panels below the second horizontal line show amino acid variants found in the experimentally infected cats atindicated time points after infection. n is the number of clones with a particular sequence at that time point.

Biological isolate AM19

In contrast to the homogeneity of the viral sequences found in the 19k1 virus stock, not a single sequence obtained from the virus stock of biological isolate AM19 was identical to the 19k1 consensus sequence (table 3a). Furthermore, for envelope regions V3 through V6 no two sequences were identical. The highest degree of variation was found in the regions V4 and V5 (table 3a, upper panel). Sequence variation was less pronounced in regions V7 through V9, where a dominant sequence in six out of nine analyzed clones was found, with only one amino acid change compared to the consensus 19k1 sequence (A741D; see table 3b, upper panel). A similar degree of

variation was observed in the OrfA protein sequences, in which 70% of the analyzed sequences were identical and the same as the 19k1 consensus sequence (table 4, upper panel). Compared to the relatively clonal population found in the virus stock of the molecular clone, the virus stock of the biological isolate contains a heterogeneous viral quasispecies.

Table 1b. Sequence variation in TM regions V7-V9 in 19k1 infected cats

							V7		
source	time	n	aa# >	650	695	708	713	741	744
19k1		10		Q	Q	V	W	A	H
cat 13	8	4		-	-	-	-	-	-
		2		-	-	-	-	-	Q
	44	8		-	-	-	-	-	-
cat 14	32	11		-	-	-	-	-	Q
		5		-	-	-	-	-	-
		1		-	K	-	-	-	-
		1		*	-	-	-	-	-
	143	1		-	-	-	-	-	-
		5		-	-	I	-	D	-
		3		-	-	I	L	D	-

Table 2. Sequence variation in OrfA in 19k1 infected cats

source	time	n	aa# >	016	055	064
19k1		12		G	Y	F
cat 13	8	12		-	-	-
	44	12		-	-	-
cat 14	32	4		-	-	-
	44	11		-	-	-
		1		-	-	S
	143	4		-	-	-
		4		E	-	-
		2		-	-	N

Table 3a. Sequence variation in SU regions V3-V6 in AM19 infected cats

				V3													V4									V5				V6	
source	time	n	aa# >	347	355	368	372	390	392	396	397	406	410	425	443	451	452	453	454	459	481	483	488	489	523	555	557	567	581	582	603
19K1				Q	G	W	D	A	S	Q	R	F	R	F	F	K	H	H	N	R	N	L	V	D	V	N	H	K	R	Q	M
AM19 (cons)				-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	D	-	-	S	-	-	-	K	Y	-	-	-	-
AM19		1		-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	T	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	K	-	-
		1		-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	T	R	-	-	-	-	-	-	-	-	-	Q	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	V
		1		-	-	*	-	-	L	-	-	-	-	-	-	-	-	-	-	K	-	-	-	N	-	-	-	-	-	-	-
cat 11	32	2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	N	H	-	-	*	-
		2		-	-	-	-	-	-	H	-	V	-	-	-	-	-	-	-	-	-	L	-	-	I	N	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	N	-	-	L	-	-	-	N	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	N	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	L	-	H	-	-	-	-	L	-	-	-	N	H	-	-	-	-
	44	2		-	-	-	-	-	-	-	-	-	-	-	-	-	R	Y	-	-	-	L	-	-	-	Q	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	A	-	-	L	-	-	-	N	H	-	-	-	-
	71	4		-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	L	-	-	-	N	H	-	-	-	-
		1		-	R	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	L	-	-	-	N	H	-	-	-	-
cat 12	32	1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-
		1		P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
		1		-	-	-	N	-	-	-	-	-	-	L	-	-	-	-	-	-	D	-	-	-	-	N	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	A	-	-	-	-	-	-	N	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	K	-	-	-	R	-	A	-	-	-	-	-	-	N	H	-	-	-	-
		1		-	-	-	N	-	-	-	-	-	-	-	-	-	R	-	A	-	-	-	-	-	-	N	H	-	-	-	-
	44	3		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2		-	-	-	-	T	-	-	-	-	-	-	-	T	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2		-	-	-	-	-	-	-	-	-	-	-	-	T	R	-	-	-	-	-	-	-	-	N	H	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	T	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	71	2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2		-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3		-	-	-	N	-	-	-	-	-	-	-	-	-	R	-	A	-	-	-	-	-	-	N	H	-	-	-	-
		1		-	-	-	N	-	-	-	-	-	-	-	-	-	R	-	A	S	-	-	-	-	-	N	H	-	-	-	-

Sequence variation during experimental infection

Four cats were infected with either the molecular clone 19k1 (cats 13 and 14) or the biological isolate AM19 (cats 11 and 12). Blood samples were drawn regularly up to 143 weeks after infection.

Molecular clone 19k1

In the animals infected with molecular clone 19k1, very little sequence variation was observed during the course of infection in both *env* (tables 1a and 1b) and *orfA* (table 2) open reading frames. Mutations were infrequent and not fixed during the course of infection and mainly occurred at single sites in individual clones. When present, the majority of the individual mutations were non-synonymous, although numbers were too low to reach statistical significance. However, multiple mutations were found in viral sequences obtained from cat 14 at 143 weeks post-infection. Although no mutations were found in envelope regions V3 – V6, for V7 – V9 eight out of nine sequences analyzed at this time point harbored multiple mutations (table 1b, lower panel) and for OrfA six out of ten clones contained single amino acid substitutions compared to the consensus 19k1 sequence (table 2, lower panel).

Biological isolate AM19

More sequence variation was found in viruses isolated from cats infected with the biological isolate AM19 compared to those infected with the molecular clone (tables 3 and 4). Amino acid substitutions were largely confined to the variable regions of the envelope glycoprotein. However, if the amino acid variants that are present in the virus stock are taken into account (tables 3 and 4; compare upper vs. lower panels), new mutations that arose during infection were rare. Similarly, mutations in the V7 through V9 regions were found sporadically, mostly in single clones. Despite the lack of sequence variation, the viral sequences obtained from cat 11 seem to indicate some sequence evolution may have occurred in the V3 – V6 region. The leucine at position 452 in the inoculum (in 60% of the analyzed sequences) was still predominant at 32 weeks p.i. (71%) but was substituted by an arginine 44 weeks p.i. (100%) and by a histidine 71 weeks p.i. (100%). To test the effect of these and other concurrent amino acid substitutions found in this animal, they were introduced into the molecular clone FIV 19k1 by site-directed mutagenesis. Virus stocks were produced and used in a virus neutralization assay using plasma obtained at different time points post infection. However, no differences in virus neutralization titers were found with these mutant viruses and plasma samples (data not shown).

Table 3b. Sequence variation in TM regions V7-V9 in AM19 infected cats

				V7								V8									V9			
source	time	n	aa# >	642	672	677	710	711	714	739	741	760	778	791	813	818	822	823	825	831	834	836	837	838
19k1				Y	H	I	L	E	R	T	A	G	G	I	R	K	Y	T	I	D	E	Q	P	Q
AM19 (cons)				-	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-
AM19		6		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	V	G	-	C	A	V	-	-	-	-	-
cat 11	32	1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	Q	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.
		1		-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P
		1		-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	M	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	44	7		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	71	5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cat 12	32	4		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-
	44	5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-
		1		-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	71	5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-
		1		-	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-
		1		-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-

Also for OrfA new mutations were rare in the AM19 infected cats, as they were only observed in one of the two cats (table 4). As can be seen in tables 3 and 4, none of the new mutations were fixed during the course of infection. Moreover, in OrfA sequences obtained from plasma samples of cat 12, no mutations could be demonstrated during the entire 143 week follow up period.

Table 4. Sequence variation in OrfA in AM19 infected cats

source	time	n	aa# >	006	017	036	050	051	059	067	068	071
19k1				V	K	I	E	P	W	W	Q	S
AM19 (cons)				-	-	-	-	-	-	-	-	-
AM19		7		-	-	-	-	-	-	-	-	-
		1		-	-	-	K	-	*	-	-	-
		1		-	-	-	-	-	-	C	H	-
		1		-	N	-	K	-	-	-	-	-
cat 11	32	9		-	-	-	-	-	-	-	-	-
		1		-	-	-	G	-	-	-	-	-
		1		E	-	-	-	-	-	-	-	-
		1		-	-	V	-	-	-	-	-	-
	44	12		-	-	-	-	-	-	-	-	-
	71	8		-	-	-	-	-	-	-	-	-
		2		-	-	-	-	T	-	-	-	A
		1		-	-	-	-	-	-	-	-	A
		1		-	-	-	K	-	-	-	-	-
cat 12	32	12		-	-	-	-	-	-	-	-	-
	44	12		-	-	-	-	-	-	-	-	-
	71	12		-	-	-	-	-	-	-	-	-

Discussion

Sequence evolution is reflecting the capacity of lentiviruses to adapt in response to the restrictions impeded by its environment, the host and its immune responses. Knowledge about viral sequence variation that occurs during infection is relevant because of its implications for e.g. vaccine development. To obtain a better understanding of the intrahost variation of FIV, we infected cats with either a FIV molecular clone or a biological isolate, which were both sequenced to assess the extent of sequence variation in the respective inocula. It was hypothesized that the cats' immune responses would drive FIV sequence evolution. The evolution of FIV

env gene was analyzed since the Env protein is a target for the humoral immune responses and known for its capacity to escape from recognition by antibodies by accumulating mutations in the variable regions especially. The replication regulating OrfA protein was chosen because it is expressed intracellularly and most likely is a target for cellular immune responses.

As expected, the viral progeny of molecular clone 19k1 was almost clonal for both envelope and OrfA sequences whereas the biological isolate AM19 contained a variety of sequence variants. AM19 sequence variation was most extensive in the V3 – V6 regions of the envelope glycoprotein, where none of the analyzed sequences in this region was identical (see table 3a).

Unexpectedly, little sequence variation was observed in circulating viruses isolated from the infected cats. Although a greater extent of variation was observed in cats infected with the biological isolate, nearly all of the variants were present in the virus inoculum used for infection of the cats and thus did not reflect the emergence of new virus variants generated in vivo. However, in two cats sequence evolution may have occurred. In cat 11, infected with the biological isolate FIV AM19, the leucine at position 452 of the envelope glycoprotein (V4 region) was substituted by an arginine and a histidine 32 and 71 weeks p.i., respectively. Although each of these amino acid variants was already present in the AM19 virus stock, it may suggest that selective pressure was at work, most likely driven by antibodies. However, exchange of these amino acids at position 452 of Env in the molecular clone 19k1 (either alone or in combination with other mutations found at the respective time points) did not affect its susceptibility to virus neutralization using homologous antisera in a virus neutralization assay based on infection of PBMC [310]. In previous studies it was demonstrated that neutralization-resistant variants emerged from less than four to more than fifteen months after infection of cats with a virus neutralization sensitive strain, a process that was driven by virus specific antibodies [19,261]. During infection of man with HIV-1 sequential neutralization escape variants emerge, the kinetics of which is dependent on the development of VN antibody responses which can vary greatly between infected subjects [1,96,254]. Interestingly, the SU (V3 – V6) consensus sequence for biological isolate FIV AM19 resembled that of molecular clone 19k32 (GenBank accession no. **M73965**) more than that of clone 19k1 [309]. It has been shown that combinations of amino acid substitutions changed the susceptibility of the molecular clones to polyclonal neutralizing antibody [312]. In the present study, the majority of the virus sequences found in cat 11 contain amino acids that rendered the 19k32 clone susceptible to neutralization by 19k1 specific antiserum [309].

Hundred forty-three weeks p.i., the virus obtained from cat 14 infected with virus 19k1 displayed multiple mutations in the transmembrane part of the Env protein (table 1b) and the OrfA protein (table 2), which may be suggestive of selective

pressure. It was reported previously that the accumulation of mutations in the FIV envelope glycoprotein coincided with the onset of progression to AIDS-like disease in the infected cat [151]. To test if a similar disease progression had occurred in cat 14, we analyzed the $CD4^+$ and $CD8^+$ T cell subpopulations and the plasma virus loads. After 71 weeks p.i., the CD4:CD8 ratio dropped below 1.0 and the percentage of $CD4^+$ T cells (of total lymphocytes) fell from 18% to 10% between 109 and 143 weeks p.i., which may suggest that this cat had progressed to AIDS.

In the present study we show that FIV displays limited sequence variation during infection of its natural host, which is in agreement with results obtained by others [151,218]. However, in the latter studies the amplicons were sequenced without prior cloning thus detecting the major virus variant only [151] or no information was available on the extent of variation in the virus inoculum, which prevented useful interpretation of the data regarding the detection of new variants after infection [218]. The relative genetic stability of FIV is in contrast with the variable nature of lentiviruses in general, which is the result of erroneously introduced nucleotides during reverse transcription. The variable nature of FIV and other lentiviruses, including HIV-1, is exemplified by phylogenetic analysis of nucleotide sequences in particular of the *env* gene obtained from a large number of viruses obtained from the population at large. This type of variation is influenced by population dynamics, involving population size, transmission rates and infection of individuals for prolonged periods of time and duration of latency [74]. Apparently, this level of sequence variation is not detected in individual hosts which has also been reported for HIV-1 infected subjects [306].

It has been reported that mutations in the RT open reading frame directly influence its fidelity [183]. To address whether any of these mutations were present in the RT enzyme of FIV 19k1, we sequenced its open reading frame. We found no mutations in the catalytic YMDD-motif [231,235], a site at which mutations, mostly associated with drug resistance, have been reported to increase HIV-1 RT fidelity [279]. However, we did observe a V148S mutation in 19k1 RT (data not shown). Interestingly, V148I is a naturally occurring mutation associated with increased fidelity of the RT of simian immunodeficiency virus (SIV). Using site directed mutagenesis it was shown that also the V148S substitution increased SIV-RT fidelity [67]. It may be speculated that the V148S substitution in the RT of FIV strain 19k1 contributed to increased fidelity compared to other lentiviruses. This possibility is highly hypothetical and further research is required to demonstrate an effect of the substitution on FIV-RT fidelity. Another possible explanation for the limited sequence variation may be that FIV has a lower replicative capacity compared to other lentiviruses. With increased replication rates, more viruses are generated per time unit, thereby allowing for a greater number of mutations to accumulate over time. For example, although HIV-2 and HIV-1 proviral loads are similar, HIV-2 replicates to lower titers in the plasma [197,267], which

correlates with slower rates of viral sequence evolution [196] and lower pathogenicity [202,266,381]. Also the low-pathogenic bovine immunodeficiency virus (BIV) displayed very little genetic variation in the *env* gene during four year of infection [43]. In contrast, it was reported that evolution of equine infectious anemia virus during infection of ponies continued during relapse of viral replication [175]. FIV seems to replicate slower than HIV-1. Although for HIV-1 and FIV peak viral loads during acute infection vary greatly from approximately 10^3 to 10^6, the peak of HIV-1 replication is reached within 2-3 weeks after infection [163,297,327] while that of FIV is reached between 1-2 months p.i. (M. Pistello, personal communication). Therefore, it could be speculated that the slower replication kinetics of FIV contribute to a lower mutation rate and a higher genetic stability over time.

A longer follow up time of the cats after infection with FIV AM19 might have exposed a higher degree of intra-host variation as a result of selective immune-pressure. Alternatively, the use of more pathogenic strains with higher replication competence, like strain FIV GL8 [144] may display a more rapid evolution.

Collectively, our data indicate that even during almost three years of infection of cats with FIV, limited sequence variation is observed, which may be related to lower replication competence than some other lentiviruses, high fidelity of its RT and/or limited immune pressure from the host. It may also mean that in certain cat populations the rapid emergence of escape mutants does not have to be taken into account, which is in favor of the development of a vaccine that could induce protective (herd) immunity in those cat populations.

7

Summarizing Discussion

In part based on
Vaccine review, accepted for publication

The present thesis largely focused on the evaluation of different strategies to develop a safe and effective vaccine against FIV infection of the cat. The development of a vaccine against FIV would not only be important for the implementation of intervention strategies for FIV infection and FAIDS in domestic cats, feline zoo collections and endangered wild felid species, but could also serve as a blueprint for the development of a human vaccine against HIV infection and AIDS. The latter would be based on the close similarities between the lentivirus infections of cats and humans (Chapter 1). Although some progress has been made in the development of HIV vaccines, the outcome of various clinical trials shows that the clinical use of a therapeutic vaccine, let alone a preventive vaccine is still quite a distant goal. The FIV-cat model for lentiviral vaccine development and evaluation with its inherent limitations (see Introduction) provides a useful system to assess the critical requirements for safe and effective lentiviral vaccines. First it offers the unique opportunity to study efficacy and safety of candidate vaccines in a natural host. Subsequently, route, dose, and nature (e.g. cell free vs. cell-associated) of challenge can be chosen to mimic different natural modes of transmission, including mucosal (sexual transmission), intravenous (drug abuse; transmission by contaminated needles), and subcutaneous/intramuscular (needle accidents) infection. Perhaps the most important asset of the FIV-cat model for lentiviral vaccine development and evaluation is, that correlates of protection or alternatively immune mediated pathogenesis or enhancement, induced by vaccination, can be studied in a natural host. The strategies followed in this thesis were partly chosen on the basis of previous studies by others and ourselves in the FIV-cat system that had shown that envelope glycoprotein based vaccine candidates not only failed to protect vaccinated cats from infection, but in certain cases even were counterproductive. They caused enhanced susceptibility to experimental FIV infection, even when vaccine induced envelope-specific (neutralizing) antibodies were present on the day of challenge [138,281,312]. Also envelope-based vaccine candidates that aimed at the induction of HIV neutralizing antibodies in humans have so far not been successful in protecting individuals with a high risk of acquiring HIV infection [92,111]. Therefore our attempts aiming at the induction of protective FIV specific immune responses have largely focused on two strategies, either alone or in combination:

- the induction of specific protective antibody responses, while avoiding the induction of enhancing antibody responses
- the induction of specific protective T cell responses.

Vaccination strategies followed

First, it was shown that with different ISCOM-based WIV and FIV subunit vaccines, high titres of both Gag- and Env-specific plasma antibodies were induced in cats (Chapter 2). The highest levels of envelope specific plasma antibodies were observed in cats that had received vaccine preparations that contained purified envelope glycoprotein derived from vaccinia recombinants that were incorporated into ISCOMs. Although FIV neutralizing antibodies were induced, none of the animals proved to be protected from challenge infection with a largely homologous FIV strain. In this context, it is important to note that VN antibodies could only be demonstrated in the CrFK cell based assay, using a FIV strain adapted to replicate in this cell line. In HIV infection of humans a similar phenomenon was described: antibodies that efficiently neutralize T cell line adapted HIV-1 strains, fail to neutralize or even enhance infectivity of primary HIV-1 isolates in primary lymphoid cell cultures [215].

We had previously reported the results of studies with FIV subunit vaccines, in which the induction of VN antibodies, only detectable in the CrFK cell based VN assay, predisposed for accelerated viremia rather than for protection against FIV challenge [312]. In the experiments reported here, cell associated virus loads, measured two weeks after challenge infection also showed a tendency toward accelerated viremia in cats vaccinated with FIV-ISCOMs. Paradoxically, three out of six cats, vaccinated with ISCOMs prepared with the supernatant of uninfected CrFK cells also proved to be viremic as early as 2 weeks after challenge, suggesting that if in this group antibody mediated enhancement had indeed played a role, antibodies to cell derived components in the virus membrane rather than "proper" virus specific antibodies were involved. Addition of bacterially expressed Gag protein incorporated into ISCOMs in the vaccine preparation seemed to counterbalance the enhancing effect induced by vaccination with purified FIV envelope glycoprotein containing vaccines. It is interesting to note that the two cats in which FIV infected PBMC could be demonstrated had developed the lowest anti-Gag response of this group at the day of challenge. Since it cannot be expected that Gag specific antibodies would have contributed to the observed protective effect, it may be speculated that the effect was mediated by Gag-specific T cell mediated immunity.

On the basis of this and several other published vaccination studies we speculated that differences in outcome between the few successful FIV vaccination experiments on the one hand [139,204,394,395] and the large number of unsuccessful vaccination experiments on the other hand [90,112,138,139,195,312,345,369] indicate that mechanisms leading to vaccine induced protective immunity are counteracted by those leading to vaccine induced enhancement.

Subsequently it was shown that antibodies directed against FIV envelope regions HV3-5 are not solely responsible for vaccine-induced acceleration of challenge infection (Chapter 3). Transfer experiments had indicated that antibodies directed against the envelope protein, after vaccination with an FIV envelope protein-ISCOM, were involved in the predisposition of cats for accelerated viremia upon challenge infection with FIV. The accelerated virus replication correlated with the presence of virus neutralising and HV3-5 specific serum antibodies on the day of challenge [312]. In addition, vaccination of cats with a peptide containing the V3 region of the FIV envelope protein predisposed the cats for enhanced virus replication which correlated with accelerated VNA titre kinetics [192]. To address the possible role of HV3-5 specific antibodies in vaccine-induced acceleration of infection, we vaccinated cats with ISCOM candidate vaccines containing either the entire FIV envelope glycoprotein [312] or the envelope glycoprotein from which regions HV3-5 had been deleted (FIV-ΔEnv-ISCOM). Plasma viral and proviral load kinetics were similar for both the FIV-Env-ISCOM and the FIV-ΔEnv-ISCOM vaccinated cats, indicating that antibody responses against HV3-5 were not solely responsible for FIV envelope vaccine-induced acceleration of infection. In contrast to the FIV-Env-ISCOM vaccinated cats, enhancement in the cats vaccinated with FIV-ΔEnv-ISCOM took place in the absence of a detectable VN antibody response, most likely caused by the absence of envelope regions HV3-5 in the vaccine preparation. Despite the absence of these regions in the vaccine preparation, FIV-ΔEnv-ISCOMs were still immunogenic, as measured by Env-specific ELISA. Furthermore, although the FIV-ΔEnv-ISCOM failed to induce VN antibodies, the vaccinated cats were apparently primed for the development of VN antibodies as such antibodies developed more rapidly after challenge infection in the vaccinated than in the control group. Alternatively, this anamnestic response could also be the result of accelerated virus replication kinetics in these animals. The antibody response induced by FIV-ΔEnv-ISCOMs may have been redirected towards regions on the envelope protein other than HV3-5 and have primed for the induction of post-challenge antibodies that neutralize FIV *in vitro* but may predispose for accelerated infection *in vivo*. Envelope specific antibody responses measured by ELISA indicated that these responses may indeed have been redirected towards other epitopes in the FIV-ΔEnv-ISCOMs vaccinated animals. The role of such antibody responses in the observed acceleration of infection remains unclear. Passive transfer of post vaccination plasma and subsequent infection could provide supportive evidence for the hypothesis that the accelerated virus replication in FIV-ΔEnv-ISCOM vaccinated cats was antibody mediated, as was shown to be likely for cats vaccinated with FIV-Env-ISCOMs, by carrying out plasma transfer experiments [312].

Collectively, the data obtained in this study do not support the notion that epitope deletion is a viable approach to improve the efficacy of candidate lentiviral vaccines. Interestingly, vaccination studies with HIV Env from which HV regions have been deleted are still ongoing [49]. In conclusion, the role of aberrant antibody responses in FIV vaccine-induced enhancement of infection is not as clear-cut as initially proposed [312].

In the majority of the thus published FIV vaccination/challenge studies, cats are challenged relatively short after the last (booster) vaccination, usually two to four weeks. Consequently, (polyclonal) immune activation has been suggested as an alternative mechanism underlying the occurrence of vaccine-induced acceleration of infection [161,281,284]. In an attempt to address this phenomenon we also challenged FIV-Env-ISCOM vaccinated cats twelve weeks after the last vaccination, i.e. a postponement of the challenge infection with ten weeks, compared to the original scheme. However, because the outcome of this challenge infection was inconclusive, a role for immune activation in FIV vaccine-induced enhancement of infection could not be accurately assessed.

Since attempts to develop a FIV vaccine based on the strategy to induce FIV neutralizing antibodies were largely unsuccessful, FIV vaccine candidates aiming at the strategy to induce protective T cell mediated immunity by the inclusion of the early, regulatory proteins Rev and OrfA as antigens, were evaluated with and without inclusion of the Env protein (Chapter 4). Bacterially expressed FIV proteins were delivered in ISCOM preparations to promote the induction of cellular immunity against early regulatory proteins of the virus, since it had been shown that the induction of cell mediated immunity against these proteins was associated with protection in both HIV-1 infection of man and a number of other lentivirus infection models [4,108,125,230,246,358]. However, none of the candidate subunit vaccines induced protective immunity, since all of the cats became viremic upon challenge infection. Although antibody responses were induced or primed for, specific cell mediated immunity could not be detected in the vaccinated cats in IFN-γ ELISPOT assays, by using PBMC and pools of overlapping peptides representing the complete open reading frames of FIV Rev and OrfA proteins. However, after infection with FIV we were able to detect IFN-γ producing cells could be demonstrated in a substantial portion of the cats, but independent of the vaccine that was used. This shows that it is possible to detect these responses by ELISPOT using pools of overlapping peptides but also that the vaccine preparations failed to substantially augment cellular immunity against their respective components. Apparently, vaccination with the vaccine candidates induced cellular immune responses with insufficient efficiency. Alternatively, it could also be speculated that FIV Rev and OrfA proteins contain a

minor number of T-cell epitopes that can be presented by a limited number of selected MHC class I molecules. Indeed, using overlapping peptides and PBMC obtained from FIV-infected cats in an IFN-γ ELISPOT assay, it was shown that OrfA harboured a limited number of T cell epitopes as compared to Gag, Env and reverse transcriptase (RT) proteins [65]. In another study in which the potential of OrfA based candidate FIV vaccines was assessed, it was shown that the induction of virus specific cell-mediated immunity was difficult to achieve and that only transient responses were observed shortly after vaccination [263]. Although the use of bacterially expressed, truncated envelope proteins did not coincide with the induction of enhancement of infection [312], the inclusion of bacterially expressed envelope glycoprotein in this study failed to induce virus neutralising antibodies and did not improve the performance of the Rev and OrfA preparations. Rather, there was again a tendency towards enhancement of susceptibility to FIV infection, albeit not statistically significant.

Collectively, the outcome of this vaccination study indicated that the use of FIV Rev and OrfA proteins either alone or in combination with envelope protein preparations did not confer protection against challenge infection with FIV in cats. Thus, although the use of accessory proteins as vaccine candidates has been successful in some animal models for lentiviral infections, their use in combination with ISCOMs as an adjuvant was not successful in the FIV-cat model. It could be speculated that other delivery vehicles or vectors may be necessary for the induction of protective immunity against FIV with these viral proteins.

The use of DNA and viral vectors as means to deliver FIV accessory proteins Rev and OrfA was therefore evaluated subsequently (Chapter 5). Cats were primed by inoculation with expression plasmids based on SFV DNA vectors and boosted by recombinant MVA vectors driving the expression of FIV Rev and OrfA proteins in a prime-boost vaccination scheme that had shown to be effective in other lentivirus vaccination/challenge models [226,238,330,367].

Despite the induction of vector-specific antibody responses, Rev or OrfA specific humoral and cell-mediated immunity could not be detected after completion of the prime-boost vaccination schedule. Therefore not unexpectedly, these recombinant vector vaccines failed to confer protection against FIV infection. Inefficient vector-mediated expression was the most likely cause of the observed vaccine failure. As suggested earlier (Chapter 4), the small size of the Rev and OrfA proteins and the corresponding limited number of epitopes that can be recognized by T lymphocytes may explain the lack of these candidate Rev and OrfA vaccine efficacy. Clearly, the hopeful results obtained with candidate vaccines based on the use of accessory proteins in primate models of lentiviral infection [40,238,330] could not be reproduced in the FIV-cat model and the data obtained in this study indicate that a combination of

SFV and MVA mediated delivery of FIV Rev and OrfA proteins is not suitable for the induction of protective immune responses against FIV.

Intrahost genetic variation

For the development of a vaccine against FIV it is important to have a good understanding of the genetic variability of the virus in the feline population at large as well as the kinetics of the genetic variation upon infection in individual cats. Although much is known about the genetic diversity of FIV in the cat population (see also Chapter 1), little is known about the latter. Therefore, the extent of sequence variation and evolution in FIV proteins Rev, OrfA as well as the envelope glycoprotein were assessed over a period of up to three years in experimentally infected cats (Chapter 6). To this end, cats had been infected either with a biological FIV isolate (FIV AM19) or with a molecular clone that was generated from the same animal (FIV 19k1) [309,312]. The Rev and OrfA genes were chosen because their proteins were postulated to be targeted by protective cellular immune responses, due to their expression early in the viral replication cycle, whereas the envelope glycoprotein was chosen for analysis as it is a major target for antibody mediated immune responses that have also been postulated to be protective. In particular VN antibodies have been shown to be involved in driving the evolution of envelope sequences during the course of infection with other lentiviruses, including HIV-1 [96,293]. To be able to identify whether variant sequences identified during infection period were truly new or had already been present in the virus stocks used for infection, the level of sequence variation was also assessed for the original inocula. The extent of sequence variation was most extensive in the AM19 stock, for which the surface subunit of the envelope glycoprotein contained not a single identical sequence among the ten clones that were sequenced. In contrast, most of the progeny of molecular clone 19k1 was clonal.

Surprisingly, the extent of sequence variation observed over the course of infection proved to be very limited for both the AM19 and 19k1 infected animals. In fact, the number of mutations occurring throughout the entire follow-up period proved to be too limited in all animals to allow statistically accurate calculations. Although a greater extent of variation was observed in cats infected with the biological isolate AM19, compared to the cats infected with the molecular clone 19k1, nearly all of the variants had already been identified in the original virus inoculum used for infection of the cats and thus did not reflect the emergence of new virus variants generated *in vivo*. A hint towards immune-driven sequence evolution was observed in one cat infected with the biological isolate. However experiments mimicking the evolution by introducing the identified amino acid substitutions into the 19k1 backbone and assessing the VN phenotypes of the resulting viruses could not confirm this.

In one of the cats the appearance of multiple substitution mutations in both the envelope glycoprotein and the OrfA protein at 143 weeks after infection coincided with the first observed clinical signs suggestive for progression towards FAIDS, and indicating that possibly the three year follow-up period had not been long enough for significant mutations to emerge in the present study.

Nevertheless, the relative genetic stability of FIV during infection, which had been identified earlier in less extensive analyses [151,218], is in apparent contrast with results published previously on FIV infection of cats [19,145,261] and on HIV-1 infection of humans [1,96,254], although the kinetics of (immune-driven) sequence variation seems to vary greatly between infected individuals. Interestingly, a V148S mutation in the RT enzyme that has been shown to increase its fidelity in SIV [67], was observed in the FIV 19k1 RT enzyme, which may be suggestive of an increased inherent RT fidelity for this virus strain, although further research is clearly needed to assess this more accurately. Alternatively, a lower replicative capacity for FIV may provide an explanation for the lack of sequence variation in this and previous studies, as reports on HIV-2 and BIV sequence variation suggest that a lower replication rate coincides with reduced pathogenicity and sequence variation [43,196,197,202,266,267,381]. However in EIAV infection of ponies it has been observed that sequence evolution was continuously occurring even during relapse of viral replication [175].

As suggested above, a longer follow up time of the cats after infection with FIV AM19 might have led to a higher degree of intra-host sequence variation. Alternatively, the use of more pathogenic strains with higher replication competence [144,397] may display more substantial levels of sequence variation during infection. On another note, limited sequence variation in FIV strains in certain cat populations could be in favor of the development of a vaccine capable of inducing protective immunity in those populations.

VACCINE-INDUCED ENHANCEMENT OF VIRAL INFECTIONS

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Abstract

Examples of vaccine-induced enhancement of susceptibility to virus infection or of aberrant viral pathogenesis have been documented for infections by members of different virus families. Several mechanisms, many of which still are poorly understood, are at the basis of this phenomenon. Vaccine development for lentivirus infections in general, and for HIV/AIDS in particular, has been little successful. Certain experimental lentiviral vaccines even proved to be counterproductive: they rendered vaccinated subjects more susceptible to infection rather than protecting them. For vaccine induced enhanced susceptibility to infection with certain viruses like feline coronavirus, Dengue virus, and feline immunodeficiency virus, it has been shown that antibody dependent enhancement (ADE) plays an important role. Other mechanisms may, either in the absence of or in combination with ADE, be involved. Consequently, vaccine-induced enhancement has been a major stumble block in the development of certain flavi-, corona-, paramyxo-, and lentivirus vaccines. Also recent failures in the development of a vaccine against HIV may at least in part be attributed to induction of enhanced susceptibility to infection. There may well be a delicate balance between the induction of protective immunity on the one hand and the induction of enhanced susceptibility on the other. The present paper reviews the currently known mechanisms of vaccine-induced enhancement of susceptibility to virus infection or of aberrant viral pathogenesis.

Introduction

Lentiviruses have infected several mammalian species including humans (human immunodeficiency virus-1 (HIV-1) and HIV-2), non-human primates (simian immunodeficiency viruses (SIV's)) and cats (feline immunodeficiency virus (FIV)), sometimes affecting a significant proportion of the host population (for reviews, see refs. 51 and 364) Despite their relatively wide distribution, the transmission of lentiviruses is generally not very efficient. After inoculation, the virus enters host target cells via interaction with one or more cellular receptors. For HIV-1, HIV-2 and SIV, CD4 is used as the primary receptor while chemokine receptors like CCR-5 or alternatively CXCR-4 are required as secondary receptor. Similarly, FIV enters its target cell using CD134 as a primary and CXCR-4 as a co-receptor. Interference with viral entry by vaccine-induced antibodies or antiviral therapy has been one of the major goals in the development of lentiviral intervention strategies. In spite of huge investments, success in the field of lentivirus vaccine development has been limited and in some cases the use of experimental lentiviral vaccines proved to be counterproductive: it rendered vaccinated subjects more susceptible to infection. Here we review reported examples of vaccine induced enhanced susceptibility to virus infection in general and lentivirus infection in particular, as well as currently known mechanisms that may underlie this phenomenon.

Antibody dependent enhancement of viral entry: lessons from non-lentivirus systems

Dengue virus

Antibody dependent enhancement (ADE) of virus infection by increasing viral entry is a mechanism that has been observed for viruses of several families and has also been shown to play an important role in the natural pathogenesis of some of these (for review see ref. 337). Probably the best-known example of ADE of infection is the *in vitro* enhancement of Dengue virus (DENV, a member of the Flaviviridae family) entry by virus specific antibodies. The genus Flavivirus, family Flaviviridae, consists of arthropod-borne viruses such as Murray Valley encephalitis virus (MVEV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and DENV. Four serotypes of DENV have been described, with multiple serotypes co-circulating in endemic areas. Infection with any of the DENV serotypes may result in a spectrum of clinical signs and symptoms, ranging from a mild influenza-like illness, known as dengue fever (DF), to the most severe forms of the disease characterized by coagulopathy and increased vascular permeability: dengue hemorrhagic fever (DHF). This may progress

to hypovolemic shock in certain patients causing dengue shock syndrome (DSS). ADE was first described in *in vitro* systems for MVEV and WNV in 1964 [127]. ADE was subsequently postulated by Halstead and colleagues based on the observation that DHF and DSS were predominantly seen in children experiencing a second infection with a heterologous DENV serotype [121,122]. They observed that the incidence of DHF and DSS peaked in two populations of young children [123]. One peak occurred in infants (aged 6 to 9 months) that were infected with a DENV serotype different from the serotype that had infected their mothers previously. The key observation was that severe disease occurred in infants whose maternal antibodies had declined to low, sub-neutralizing levels. The other peak was observed in young children who had experienced an earlier, usually mild or subclinical, infection and were later infected with a different DENV serotype. These observations led to the conclusion that pre-existing immunity against DENV could predispose individuals for a more serious infection with a heterologous serotype of DENV and did not afford protection against disease. Later, several epidemiological studies provided circumstantial evidence for the role of pre-existing humoral immunity in the pathogenesis of DHF [37,114,166,167,296]. *In vitro* experiments showed that DENV infection could be enhanced using polyclonal antisera raised against heterologous DENV serotypes [121,122]. Furthermore, it was shown that administration of DENV-specific maternal antibodies enhanced the severity of DENV infection of rhesus macaques [120]: monkeys infected in presence of anti-DENV antibody developed higher levels of viremia and for a longer period than the control monkeys.

Although many of the details regarding Fc receptor γ (FcγR) mediated entry of DENV are still unclear, FcγRIa and FcγRIIa were shown to play an important role [26,32,188,292,298]. FcγRIIa was more efficient than FcγRIa in enhancing DENV infection of FcγR-transfected cells *in vitro* [292]. The role of FcγRIII in ADE of DENV infection remains unknown, but is likely to be involved as well. The expected result of ADE for viral infection would be an increased viral load [185,365,377], most likely caused by infection of a higher number of susceptible cells. Alternatively, FcγR mediated entry may modulate the antiviral immune response [45]. Recent studies with Ross River virus and DENV showed that entry via the FcγR pathway could suppress the expression of antiviral genes and enhance IL-10 production in mononuclear cells *in vitro*. In contrast, entry via the usual cellular receptor did not affect the induction of antiviral effector mechanisms [186,198]. Although the pathogenesis of DHF/DSS has not been elucidated, the ADE of infection hypothesis remains a significant concern in the development of safe and effective vaccines.

Feline Corona Virus

Feline Corona Virus (FCoV) infection of cats is usually mild but can also lead to the development of a chronic immune mediated disease with a high fatality rate, called feline infectious peritonitis (FIP). FIP develops upon the occurrence of spontaneous mutations in certain regions of the FCoV genome that change the cell tropism of the virus, allowing it to replicate in macrophages. This is considered to be largely responsible for FCoV infection to develops from a relatively apathogenic into a chronic, immune mediated disease. FIP disease manifests itself with a variety of clinical symptoms due to the infection of multiple organs, often including the central nervous system [126]. Early experiments had shown that transfer of plasma containing high-titred FCoV specific antibodies to naïve kittens rendered these animals susceptible to more rapidly developing FIP than kittens receiving FCoV negative serum [379]. ADE of infection was subsequently shown to be mediated by antibodies directed against the viral spike (S) protein. Immunization with recombinant vaccinia virus preparations expressing the FCoV-S protein resulted in the induction of S-specific antibody responses and low-level neutralizing antibody titers and lead to an enhanced susceptibility to challenge infection in young cats [366]. During FIP, FCoV primarily targets cells of the macrophage/monocyte lineage and ADE is thought to occur through binding of antibody-bound FCoV to the Fc receptor on the cell membrane [52,130,233]. Collectively, these data show that ADE mediated by S-specific antibody and increased viral entry into monocytes/macrophages are at the basis of the enhanced susceptibility of pre-immune cats to develop FIP.

Respiratory Syncytial Virus

Enhanced respiratory syncytial virus (RSV) disease and atypical measles were observed in children who had been vaccinated with formaldehyde inactivated and alum adjuvanted candidate vaccines in the sixties upon exposure to wild type RSV or measles virus, respectively, later in life [264]. It has long been speculated that an aberrant antibody response against the viral glycoproteins was at the basis of this phenomenon. Although indeed the neutralizing serum antibody responses induced by these vaccine candidates were relatively weak, short-lived and resulted in a rapid renewed susceptibility to infection after vaccination, other mechanisms than ADE could have been at the basis of the observed immunopathology [221,222]. Besides anomalous antibody responses, there are several differences between the immunological response that is induced by either formaldehyde inactivated vaccines or natural infection that could explain the observed enhancement phenomenon: absence of specific cytotoxic T cell responses, immune complex deposition in infected tissues, increased specific proliferative $CD4^+$ T lymphocyte responses, and a bias

of the specific immune response towards a Th2 phenotype [66,234]. This was largely concluded on the basis of immunization and infection data generated in preclinical studies using mouse and macaque infection models for these viruses. Alternatively, the generation of carbonyl groups through the formaldehyde treatment of the vaccines could have contributed to the increased sensitivity to infection [211].

Human metapneumovirus

Recently, it was shown in a macaque model that formalin-inactivated human metapneumovirus (HMPV, also a member of the Paramyxoviridae family) vaccines have the same propensity to predispose for immune-mediated disease as inactivated RSV and MV vaccines: FI-HMPV-primed monkeys developed eosinophilic bronchitis and bronchiolitis upon challenge, which like in the RSV and MV models is indicative of a hypersensitivity response [61]. Collectively, these data provide us with animal models for enhanced paramyxovirus disease, which are useful to screen new generation candidate vaccines at an early stage for the potential induction of enhanced sensitivity to infection.

Antibody dependent enhancement of lentivirus entry

Also after vaccination of cats with candidate FIV vaccines enhanced susceptibility rather than protection against infection was observed [138,192,312]. In one study, passive transfer experiments were carried out which demonstrated that antibodies were probably responsible for the observed enhancement of infection [312]. Similarly, vaccination of horses against equine infectious aneamia virus (EIAV) with a recombinant S protein induced enhancement of subsequent infection, although the results of *in vitro* ADE assays did not correlate with the observed enhancement of infection [124,156,273,274,376] (see below).

Already in the early days of HIV research Robinson and Montefiori described that *in vitro* infectivity of the virus could be enhanced by virus specific antibodies [289]. Multiple mechanisms have been described that may cause or contribute to ADE in HIV infection. Virus that is complexed with antibodies may be captured and internalized by FcR [136,338] or complement receptor (CR) [97,333]. This process may or may not bypass the natural route via CD4 and a chemokine receptor depending on the experimental conditions [97,136,255,337-339,352]. In addition, the receptors may provide an activation signal to the cell after binding the virus-antibody complex, which could support virus endocytosis and increase virus production [55].

Enhancement independent of FcR and CR may also occur. Neutralizing antibodies, but also soluble CD4, can enhance NSI/R5 virus infectivity [300,331] by inducing

conformational changes in the viral envelope [118,332] and bringing the envelope in proximity of the CCR-5 co-receptor.

In general, prolonged contact of the virus and the target cell will increase the chance that receptor binding and subsequent fusion will occur. This may also be accomplished through the deposition of antibody-complement complexes on the cell, independent of CR capture, via the formation of fibrils [220,389]

Also for SIV infection of macaques ADE of infection has been described. In sera from monkeys infected with SIVmac251, ADE could be demonstrated, while this was not observed in macaques that were vaccinated with HIV-2 envelope preparations [173]. Furthermore, it has been reported that plasma obtained from SIVmac251 infected animals enhanced SIVmac infection of a human $CD4^+$ cell line, which was dependent on the presence of complement [213], in a manner similar to the complement-mediated ADE activity observed with HIV-positive human sera in *in vitro* experiments described above [289,290,391].

Enhancement of HIV replication after activation of cell-mediated immunity

Lentiviruses replicate best in activated cells of the immune system and systemic activation of the immune system generally results in enhanced virus replication, e.g. during opportunistic infections [236,362,374,375]. The higher number of activated target cells, i.e. $CD4^+$ T cells and $CD4^+$ cells of the myeloid lineage, most likely accounted for the viral bursts that had been observed. For example, bacterial products may activate $CD4^+$ T cells via toll-like receptor (TLR) 2 and possibly also TLR-4, 5 and 7 [343]. Direct intracellular interaction of replication enhancing molecules from other viruses (e.g. herpesviruses) and HIV has been shown in *in vitro* studies [241]. Given the relatively low chance that two different viruses would replicate in the same cell *in vivo*, the contribution of this mechanism will not be of great importance to the overall virus production [22].

Vaccination of HIV-1 infected individuals against other pathogens may [323,324,370] or may not [68,94] result in increased HIV-1 replication. Repeated T cell activation in SIV-infected monkeys also shortened the survival of the animals [371]. Activation of the immune system can increase the number of activated $CD4^+$ T cells, which are more susceptible to infection than resting T cells. In addition, augmented TNF-α production may be implicated in increased viral loads [370]. Infection of leukocyte adhesion molecule leukocyte function-associated antigen (LFA)-1 expressing cells may be

further increased by incorporation of intercellular adhesion molecule (ICAM)-1 into the virus particles that bud from activated T cells [28,129,287].

In addition to activated $CD4^+$ T cells there may also be a role for activated macrophages. It has recently been reported that herpes virus infection of mice can protect against Listeria monocytogenes and Yersinia pestis pathogenesis via activated macrophages [14]. In contrast, CMV infection of humans may lead to an increased susceptibility to infection with HIV-1 [199,212].

Dendritic cells are key players in generating immune responses. Many DC express DC-SIGN, a C-type lectin that can capture HIV through its Env protein [103]. HIV bound to DC-SIGN may follow different pathways leading to virus destruction (and MHC-restricted antigen presentation) or to infection. Infection of mature DC in *cis* results in low level virus production [63]. More interestingly, infection may also occur in *trans*, when the DC delivers the infectious particle to T cells that interact with the DC [103,104]. In this regard, mature DC have been shown to increase transmission of both R5 and X4 viruses to T cells [10,69,115,157]. The precise involvement of DC-SIGN in this process is still unclear[24]. Regardless of the molecules involved, HIV infection of T cells is greatly enhanced when mature DC deliver the virus. DC may act as a vehicle that, like a "Trojan horse", delivers the virus to an environment of activated T cells. The immunological synapse that forms between DC and T cells may further facilitate infection of those T cells. In addition, virions are less susceptible to inactivation when associated with DC [63].

Lentivirus candidate vaccines that have predisposed for enhanced susceptibility to infection

FIV vaccine candidates

Since its discovery in 1986 [251], numerous attempts to develop an effective and safe vaccine against FIV have been made [84,356,357]. Recently, a whole inactivated cell vaccine containing two FIV subtypes has been licensed in the US [356,396]. The effectiveness and breadth of this FIV vaccine are still subject to debate [77]. Passive and adoptive transfer studies suggested that both virus neutralizing and cellular immune responses are at the basis of the reported efficacy [396]. Nevertheless, virtually all FIV vaccines that have been evaluated so far failed to induce protective immunity and several induced increased susceptibility to infection.

The first report on enhancement of infection after vaccination against FIV dates from 1992 [138]. Cats vaccinated with ISCOM preparations containing either purified FIV particles or recombinant FIV Gag protein, as well as cats vaccinated with

formaldehyde fixed infected cells became viremic 2 to 3 weeks earlier than control cats. Furthermore, all FIV vaccinated cats became viremic upon challenge, which was not the case for the control cats in this experiment. All the vaccines used were shown to be immunogenic, although Env-specific, virus neutralizing antibody responses were only detectable on the day of challenge in the cats vaccinated with the infected cell vaccine. In another study, vaccination with a synthetic peptide representing a linear epitope in the V3 loop of the FIV envelope protein, with Freund's adjuvant, induced virus neutralizing antibodies and predisposed cats for accelerated virus replication upon challenge infection [192].

In another study with ISCOM-based vaccines containing several FIV envelope glycoprotein preparations, all the cats that received eukaryotically expressed Env glycoprotein developed Env-specific, virus neutralizing antibodies. These cats became viremic at least two weeks earlier than control cats [312]. The increased susceptibility to infection was also seen in naïve cats after transfer of plasma obtained from vaccinated cats. Collectively, these results suggested that FIV envelope specific antibodies were involved in this vaccine-induced enhancement of infection. In particular antibodies to the V3-V6 regions of the envelope protein were associated with the observed enhancement of infection. In a follow-up study, recombinant envelope protein was prepared from which the part encompassing the region between V3 to V6 was deleted [147]. Vaccination with this engineered protein still predisposed for increased susceptibility to infection and it was concluded that also antibodies directed to epitopes outside the V3-V6 region were able to cause enhancement of infection.

To attempt immunizing cats with an antigen preparation that mimics the natural conformation more closely, the use of formaldehyde fixed FIV infected autologous PBMC was evaluated [161]. Despite the induction of Gag and envelope specific antibodies, vaccinated cats were not protected from infection and again were more susceptible to infection than control cats immunized with fixed uninfected PBMC. In another study using formaldehyde inactivated FIV vaccines which induced no or poorly virus neutralizing antibody responses, enhancement of infection after a low-dose challenge infection was observed [109].

In a study by Richardson et al., general immune activation was suggested as an alternative mechanism for the induction of vaccine-mediated increased susceptibility to FIV infections [281]. Vaccination with DNA from which the FIV *env* gene was expressed induced no or weak Env-specific antibody responses and predisposed for enhancement of infection [281]. It was speculated that the immunization increased the target cell population for FIV infection, through a mechanism similar to that described after heterologous vaccination of HIV-1 infected subjects, as described above. The enhancement of FIV infection was associated with increased susceptibility of lymphocytes obtained after vaccination to *ex vivo* FIV infection and the induction

of Env specific T-helper cell responses [284]. Collectively, these results suggest that after FIV vaccination in addition to ADE also other mechanisms may contribute to increased susceptibility to FIV infection. Schwartz suggested that the induction and expansion of HIV-1 specific $CD4^+$ cells through vaccination might constitute a serious confounding factor in HIV vaccine development [301]. Indeed, it has since been shown that HIV-1 preferentially targets HIV-1 specific $CD4^+$ cells in infected individuals [71].

Likewise, enhancement of FIV infection by immune activation may result from the activation and expansion of $CD134^+$ cells [305]. CD134 – the primary receptor for FIV – is a T-cell activation marker and a co-stimulatory molecule and its expression is strictly confined to $CD4^+$ T-cells [386]. With $CD4^+$ T-cells constituting the major target for FIV early in infection [64,85], expansion of this subpopulation (through e.g. vaccination) would provide the virus with an ideal opportunity for its replication [57]. Furthermore, the FIV coreceptor CXCR4 molecule is expressed on activated T-, B- cells and monocytes [385]. It was demonstrated that increased expression of CXCR4 in cell lines resulted in enhanced FIV replication *in vitro* [58]. Recently, using flow cytometric analysis of cell populations *ex vivo*, a clear positive association between CXCR4 expression and FIV infection was demonstrated, although results also suggested the existence of an CXCR4-independent mechanism of infection [353].

In summary, selective expression of CD134 induced by vaccination against FIV or other pathogens may support FIV replication during the early stages of infection in lymphocytes that are essential for sustaining memory immune responses. This parallels the targeting of R5 strains of HIV to memory $CD45RO^+$ T cells that exclusively express CCR5 early after infection [387]. Activation and subsequent signalling via upregulated CXCR4 expression may induce cellular changes favouring further viral integration and replication in the lymphatic system, thus facilitating dissemination of FIV infection.

Recent studies suggest that feline dendritic cells (feDC) play a role in the transmission of FIV comparable to that of human DC and HIV infection. It was shown *in vitro* that feDC can increase FIV infection of resting cells, but in particular of activated $CD4^+$ T-cells [321,363], which is reminiscent of *in vivo* results obtained for HIV and SIV [41,115,398]. Early studies showed that FIV can interact with C-type lectin receptors, like human DC-SIGN, and that this interaction facilitated FIV infection in *trans* [59], similar to the mechanism described for HIV-1 (see above). Furthermore, it was shown that feDC expressed the primary CD134 receptor and, to a lesser extent, the CXCR4 co-receptor. Although less efficient than lymphocytes, feDC support productive FIV replication [276]. feDC may contribute to the infection of (activated) $CD4^+$ T-cells by immediate transfer involving exosomes, endolysosomal pathways or transfer of *de novo* generated FIV particles during productive infection of DC. Collectively, these

data suggest an additional role for feDC in vaccine-induced enhancement of infection in that they might facilitate the infection of (vaccine-induced) activated $CD4^+$ T cells.

EIAV vaccine candidates

Vaccination of ponies against EIAV with inactivated whole virus or envelope subunit vaccine preparations completely protected the animals from homologous challenge infection but failed to induce protective immunity against infection with a heterologous virus strain. However, in the animals vaccinated with inactivated whole virus, levels of viral replication after challenge with a heterologous EIAV strain were merely suppressed. Moreover, 40% of ponies vaccinated with the envelope subunit vaccine exhibited signs of enhanced disease upon heterologous challenge infection [156]. The use of recombinant envelope protein failed to afford protection against infection with a homologous virus and predisposed for enhanced virus replication and disease in animals after heterologous challenge infection [376]. The vaccine-induced Env-specific antibody titers did not correlate with the outcome of challenge infection and the mechanism underlying the observed enhancement of EIAV infection remains to be elucidated [124,273,274].

SIV vaccine candidates

Vaccination of rhesus macaques with attenuated varicella–zoster virus vaccine expressing SIV Env elicited non-neutralizing Env-binding antibodies and little if any Env-specific cytotoxic T lymphocyte responses. Upon challenge infection with a heterologous SIV strain increased levels of SIV replication, more rapid $CD4^+$ lymphocyte depletion, and accelerated progression towards AIDS was observed in these animals compared to control animals. This correlated with increased $CD4^+$ T cell proliferation immediately after SIV challenge, which most likely was the result of an anamnestic response to SIV antigens. This indicates that activation of the virus-specific $CD4^+$ T cells in the absence of an adequate $CD8^+$ T cell response may enhance virus replication and disease [325]. These findings were corroborated by the increased viral set point and accelerated disease progression in macaques treated with IL-15, which activates $CD4^+$ T cells [219]. The concomitant enhanced $CD8^+$ T cell responses against SIV were not capable of containing the virus replication.

Similarly accelerated disease progression was observed in macaques vaccinated with defective provirus or recombinant Sendai expressing Gag. Although the latter approach reduced viral loads during acute infection, some of the vaccinated animals developed increased viral loads during chronic infection and progressed more rapidly towards AIDS [194,203]. The increased viremia was independent of an observed

SIV-specific $CD8^+$ T cell response, but correlated with the decline of SIV-specific $CD4^+$ T cells [194].

HIV vaccine candidates

Clinical trials in humans involving virtually all the approaches that are currently available for viral vaccine development have been carried out in the past decade. However, most of these were phase I or II trials in which due to the design of the trial enhancement phenomena would not be encountered. More recently a number of phase III trials have been conducted in seronegative volunteers at high risk for HIV infection, using monomeric gp120 preparations. The outcome of these trials was far from encouraging, with very limited induction of HIV neutralizing antibodies and no evidence for protection [92,111]. However in these trials so far no indication for ADE or other forms of enhanced susceptibility were found.

More recently, a phase IIb efficacy trial was conducted in a similar high-risk population, with attenuated recombinant adenovirus-5 (Ad5) candidate vaccines expressing HIV gag, pol and nef genes. Also in this trial no protective efficacy was observed. In contrast, a significant trend towards an increased HIV-1 infection rate was observed in volunteers that had high pre-existing antibody titers against Ad5 (> 200), compared to individuals with low pre-existing Ad5-specific titers (< 200) [50]. Due to the setup of this trial and the complexity of the required statistical analyses, a thorough assessment of possible confounding factors is pending (http://www.hvtn.org/science/1107.html, presentation: "STEP Trial: Efficacy Analyses"). Although the two categories of vaccinees were defined on the basis of their antibody titers against Ad5, it is unclear how Ad5 specific antibodies could enhance susceptibility to HIV infection. It has been proposed that the Ad5 antibodies would re-direct the vaccine to other cell types and that this could result in a different type of immunity [216]. How this would result in enhanced transmission of HIV in these vaccinees remains to be elucidated. Another hypothesis would be that the antibody levels in the persons naturally infected with Ad5 reflect the overall immunity against this virus. This would imply the presence of increased numbers of Ad5 specific memory $CD4^+$ T cells. These cells would be readily re-activated after vaccination with the recombinant Ad5-vaccine and thus create "an abundant pool of susceptible cells" as targets for incoming HIV in the Ad5 seropositive persons.

Yet another hypothesis would be that the groups with the highest Ad5 titers contain a larger percentage of people that have recently been in contact with Ad5, either as a first infection or as a re-infection. This would lead to the presence of activated memory T cells, including $CD4^+$ cells, which may contribute to increased susceptibility towards HIV infection. If any of these two last hypotheses would be

valid this would prohibit the use of many viral and other vectors as carriers for lentiviral antigens in vaccines since many of the attenuated vectors that are being exploited to date are based on, or closely related to viruses that commonly circulate in the human population. Cross-reactive immune responses will be frequently present and the resulting activated immune system may lead to enhanced HIV replication rather than provide protection. This could also pose problems in Ad5 naïve persons since infection with the wild type Ad5 virus upon vaccination with the Ad5 vector virus, will result in enhanced immunity to the Ad5 virus and an enlarged pool of activated $CD4^+$ T cells. This pool might increase the susceptibility for HIV if co-infections occur in areas where both HIV and Ad5 are endemic [117].

Collectively, the data obtained in HIV-1 candidate-vaccine clinical trials have been disappointing so far. None of the candidate vaccines tested did afford protection and even worse, some even enhanced susceptibility to HIV infection. A better understanding of the mechanisms underlying vaccine induced protection or enhancement, or in other words correlates of protection or immune pathogenesis, is urgently needed to further advance the field of HIV vaccine development.

Conclusion

Vaccine-induced enhancement of susceptibility to virus infection or aberrant pathogenesis of virus infections have been documented for infections by members of several virus families and is therefore not unique to lentiviruses.

Although identifying a single responsible mechanism for each virus/host relationship is difficult, in this review an attempt was made to identify and define the potential mechanisms involved in these phenomena (see Table 1). Firstly, ADE plays an important role in the vaccine-induced enhancement of FIV and FCoV and possibly Dengue virus infections. Secondly, a mechanism involving immune activation, mainly via activated CD4 memory T cells (not necessarily virus specific), is seen in some lentiviral systems. Activated DC may play an additional role in this mechanism in the case of HIV and possibly also FIV. Thirdly, TH2 biased and/or aberrant T cell respones often involving eosinophilia may be involved in the observed vaccine-induced enhanced disease in paramyxovirus infections such as RSV and (atypical) MV.

Vaccine-induced enhancement of infection or disease pathogenesis has been a major stumble block in the development of certain flavi-, corona- and paramyxovirus vaccines and also the recent failures in the development of a safe and effective vaccine against HIV can at least in part be attributed to the induction of enhancement rather

than the induction of protection towards virus replication by the current vaccine candidates. There may well be a delicate balance between these two outcomes and the final result of vaccination is most likely determined by the sum of these parameters. Other confounding factors that may be involved include type of candidate vaccine used, viral and/or host factors, co-infections and time after vaccination. Research specifically aimed at the identification of the mechanisms that lead to either protection or enhancement would greatly stimulate our ability to design safe and effective vaccines against lentivirus infections, and more specifically an HIV vaccine.

Table 1. Mechanisms of enhancement of susceptibility to virus infection or of aberrant viral pathogenesis mediated by pre-existing immunity

Mechanisms		Virus families (-viridae)			
		Flavi-	Corona-	Paramyxo-	Lenti-
Humoral					
	ADE	DENV WNV MVEV*	FCoV	MV (?) RSV (?) HMPV (?)	HIV* SIV FIV EIAV
Cellular					
	CD4 activation				HIV SIV FIV
	DC/trans				HIV SIV FIV*
	Aberrant T cell response			MV (?) RSV (?) HMPV (?)	

(?) = mechanism unknown/ambiguous
* = in vitro
Abbreviations: ADE: antibody dependent enhancement; DENV: Dengue virus; WNV: West Nile virus; MVEV: Murray Valley encephalitis virus; FCoV: Feline Corona virus; MV: measles virus; RSV: respiratory syncytial virus; HMPV: human metapneumovirus; HIV: human immunodeficiency virus; SIV: simian immunodeficiency virus; FIV: feline immunodeficiency virus; EIAV: equine infectious anaemia virus; DC: dendritic cells

Conclusions of the thesis

The field of vaccine development against lentivirus infections in general, and against HIV/AIDS in particular, has not been very successful in spite of all the initial expectations raised in the eighties for the development of an HIV/AIDS vaccine, the numerous efforts, and the huge investments in research and development: to date no safe and effective vaccines are available. There may be one exception, as there is a registered vaccine against FIV infection of cats. Induction of protective immunity against homologous and heterologous FIV strains has been reported [271,393], although in another challenge system vaccination of cats seemed to result in increased susceptibility to a heterologous, virulent FIV strain, rather than in protection [77]. Be it as it may, the effectiveness of this vaccine and the breadth of protection it would induce still seem to be a matter of debate.

The major problem is that even today the precise correlates of protection from infection or disease progression and perhaps equally important correlates of enhancement of susceptibility, are still largely unknown. Studies on correlation of immune parameters with lack of disease progression in infected humans and animals have provided us with certain leads like the possible protective effects of certain VN antibody and T cell responses. In addition, T cell depletion studies in macaques have shown that certain T cell responses are involved in reducing progression of disease [299]. While transfer studies using plasma and lymphocyte subpopulations from vaccinated cats have demonstrated that there is indeed a role for both antibody and T cell mediated immune responses in the induced protection [357], a more detailed analysis of the specificities of these responses is required. Once these specificities are known, the development of improved vaccines specifically tailored to induce these responses can be pursued more accurately. Unfortunately, vaccination studies aiming at the evaluation of the role of specific T cell responses in protecting cats from FIV infection or associated disease progression, can be seriously hampered by technical problems as shown in this thesis. Initially the development of assays to study these responses was hampered by the lack of reagents specific for the feline system. Such reagents are now becoming more readily available, e.g. for the development of IFN-γ ELISPOT assays that have shown to be particularly useful for the identification of potential FIV targets for cellular immunity [65,315]. Furthermore, the development of assays that do not depend on the availability of species specific reagents, like novel generation CTL assays [25,359,360] will also facilitate the identification of correlates of protective cellular immunity and, thus, the more rational design of new candidate vaccines. Obviously the identification of viral targets for vaccine induced antibody and T cell immunity is only the first step. The adequate presentation of the targets to the immune system

is the next challenge. In this light it is important to realize that even though the results obtained by vaccination of cats with regulatory proteins described in chapters 4 and 5 seem to suggest that these proteins are not suited as targets in FIV vaccine development, other delivery and/or adjuvant systems may improve the performance of such candidate vaccines.

For different purposes, different challenge models may be required. From an HIV vaccine evaluation perspective, protection from infection, or more realistically from development of disease, should first of all be achieved upon mucosal challenge and subsequently also upon parenteral or intravenous challenge. Although all these challenge routes can be explored in the FIV-cat model, a challenge model that more closely resembles contact transmission among cats in field situations would be most relevant for studying the efficacy of FIV vaccines for the cat. It is likely that challenge doses in the latter case are lower than those used in a majority of vaccination studies performed to date. Therefore, research into FIV challenge systems mimicking natural transmission "in the field" would be of great importance for the accurate assessment of veterinary candidate FIV vaccines. The risk that candidate FIV vaccines would be discarded on the grounds of an inaccurate challenge infection model should be minimized. On the other hand, this argument has also been used for conducting clinical trials in humans with candidate HIV vaccines that had not shown efficacy in animal challenge models. So far these candidate vaccines did not induce any protection against HIV infection in "the field".

Paradoxically, pre-clinical and clinical vaccine evaluation studies aiming at the induction of antibody and T cell responses against the respective lentiviruses that have shown to correlate with lack of disease progression have at best resulted in limited protection against what may be called "wimpy" challenge viruses.

Unfortunately there are numerous examples of complete lack of induced protection and even enhanced susceptibility to lentivirus infection after vaccination. Especially the field of FIV vaccine development has been confronted with vaccine induced enhanced susceptibility to infection. Although it is not clear whether indeed enhancement is more likely to occur in cats, as it has e.g. also been shown after vaccination with certain feline coronavirus vaccines (see above), a better understanding of the mechanisms underlying the observed enhancement induced by FIV vaccination may be important for the whole field of lentivirus vaccine development, as actual protection is probably the result of the sum of two phenomena: vaccine induced protection and enhancement. A curious paradox lies in the identification of T cell activation marker CD134 as the primary receptor of FIV. This may have serious implications for vaccine development - the cells that

are needed to induce and sustain vaccine-mediated immune responses are not only the primary viral targets upon infection, their function as orchestrators of the host's antiviral immune response may result in doing the exact opposite i.e. facilitating viral dissemination during acute infection. Although mechanistically different from enhancement models observed with HIV, experiments designed to study vaccine-mediated stimulation of these cells should yield answers that are of importance for both FIV as well as HIV vaccine development. These experiments should not be limited to the use of FIV vaccines but also look into the possibility that increased susceptibility to FIV replication after heterologous vaccination might constitute an additional concern. The use of candidate lentiviral vaccines presented by viral or bacterial vectors to which the vaccinees are not naïve, may be a similar reason for concern.

Finally it may be concluded that, although there are some fundamental differences between the pathogenesis of FIV infection in cats on the one hand and HIV infection in humans on the other, it should be stressed that there are even more similarities. Vaccine development for both infections has been confronted with quite similar challenges that are still largely unresolved. Therefore, given the advantages of working with the FIV-cat model over the other lentivirus models, it is important to continue and intensify studies aimed at the elucidation of the pathogenesis of FIV infection of the cat, and the associated correlates of immune mediated protection and enhancement.

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Nederlandse Samenvatting

Het onderzoek beschreven in dit proefschrift heeft zich voornamelijk gericht op het evalueren van verschillende benaderingswijzen voor het ontwikkelen van een veilig en effectief vaccin tegen feline immuundeficiëntie virus (FIV) infectie bij katten. Dit is niet alleen van veterinair belang als interventiestrategie tegen de verspreiding van FIV en AIDS bij katten, de bevindingen zouden tevens van groot belang kunnen zijn voor de ontwikkeling van een vaccin tegen humaan immuundeficiëntie virus (HIV) en AIDS bij de mens. De uitkomsten van klinische trials met verschillende HIV vaccins hebben, ook recentelijk nog, laten zien dat het ontwikkelen van een therapeutisch vaccin, laat staan een preventief vaccin, nog altijd een grote uitdaging vormt waarvoor een lange adem vereist is. Met het FIV-katten model voor de ontwikkeling en evaluatie van lentivirale vaccins is een relevant model voorhanden om de voorwaarden waaraan een dergelijk vaccin zou moeten voldoen te toetsen. Allereerst biedt het het unieke voordeel dat lentivirale prototype-vaccins kunnen worden getest in een natuurlijke gastheer met een infectieverloop en een gefaseerde ziekte-ontwikkeling die sterke overeenkomsten vertonen met HIV en AIDS bij de mens. Daarnaast kunnen zaken als infectieroute, vaccindosering en formulering gevarieerd worden al naar gelang de gewenste interventiestrategie voor verschillende transmissieroutes: mucosale infectie (seksuele overdracht), intraveneuze infectie (overdracht door besmette naalden bij bv. intraveneus drugsgebruik) en subcutane dan wel intramusculaire infectie (overdracht bij bv. prikaccidenten). Een ander belangrijk aspect van het FIV-kattenmodel is dat in een natuurlijke gastheer van een lentivirus onderzocht kan worden welke (vaccin gemediëerde) afweerreacties betrokken zijn bij bescherming als ook welke afweerreacties averechts zouden kunnen werken (bijv. vaccin gemediëerde versnelde infectie of verhoogde gevoeligheid voor infectie).

De in dit proefschrift geteste vaccins zijn mede gekozen op basis van eerdere studies in onze en andere groepen waarbij gevonden werd dat vaccins op basis van het envelop eiwit van FIV niet alleen onvoldoende effectief bleken om FIV infectie van katten te voorkomen maar waarbij gevaccineerde katten zelfs gevoeliger bleken voor infectie: katten gevaccineerd met envelop preparaten werden na experimentele infectie eerder virus-positief dan dieren die niet tegen FIV gevaccineerd waren, soms zelfs terwijl antilichamen tegen het virus aantoonbaar waren op de dag van infectie. Ook in klinische onderzoeken waarin de werkzaamheid van vaccins gebaseerd op HIV envelop in mensen werd getest is de effectiviteit in populaties met een hoge kans op besmetting niet aangetoond. De in dit proefschrift beschreven vaccins zijn dan ook gekozen op basis van een tweetal doelstellingen:

- Het opwekken van virus-specifieke, beschermende antilichaamresponsen terwijl antilichaamresponsen die betrokken zijn bij een verhoogde gevoeligheid voor infectie voorkomen dienden te worden
- Het opwekken van virus-specifieke, beschermende T cel-responsen.

In hoofdstuk **2** is de effectiviteit van zowel geïnactiveerd virus als van subunit vaccins onderzocht. Alle geteste vaccins werden geadjuveerd door inbouw in immuunstimulerende complex (ISCOM)-structuren. Aangetoond werd dat de vaccins hoge antilichaamtiters opwekten tegen zowel de Gag als envelop (Env) structurele eiwitten waarbij de hoogste responsen gevonden werden in de katten die met Env preparaten werden gevaccineerd die verkregen waren met behulp van een recombinant vaccinia virus expressiesysteem. Echter, hoewel FIV neutraliserende antilichaamresponsen meetbaar waren in deze katten, bleken ze niet beschermd te zijn tegen infectie met een grotendeels homologe FIV stam. Interessant in deze context is het feit dat de virus neutraliserende antilichaamresponsen alleen gemeten konden worden in een systeem gebaseerd op infectie van CrFK cellen waarbij een FIV stam wordt gebruikt die aangepast is om in deze cellijn te kunnen repliceren. In een systeem gebaseerd op infectie van primaire T cellen met een primair FIV isolaat konden geen virus neutraliserende antilichamen worden aangetoond. Bij virus neutralisatie van HIV-1 infectie is een soortgelijk fenomeen beschreven.

In een eerder experiment met soortgelijke Env preparaten was aangetoond dat deze de katten meer ontvankelijk maakten voor infectie met FIV en dat deze predispositie correleerde met de aanwezigheid van virus neutraliserende antilichamen in het CrFK systeem. Ook in het huidige experiment suggereerden de gemeten virustiters in de lymfocyten van geïnfecteerde katten dat er wellicht ook in dit geval sprake was van een verhoogde gevoeligheid voor infectie, al zouden naast Env specifieke antilichamen dan ook antilichamen tegen cellulaire componenten van het vaccin een rol hebben kunnen spelen. Dit omdat de helft van de katten gevaccineerd met een controlepreparaat dat slechts celmateriaal en geen virus bevatte, virus ook eerder meetbaar was. Toevoeging van Gag, verkregen middels een bacteriëel expressiesysteem, leek de averechtse werking van deze Env vaccins tegen te gaan. Aangezien het onwaarschijnlijk is dat Gag specifieke antilichaamresponsen virus neutraliserende werking hebben, werd gesuggereerd dat Gag specifieke T cel-responsen een rol zouden hebben gespeeld bij dit beschermende effect.

In hoofdstuk **3** is in meer detail onderzocht hoe en welke Env specifieke antilichaamresponsen betrokken zouden kunnen zijn bij eerdergenoemde vaccin gemediëerde verhoogde gevoeligheid voor FIV infectie. Aanleiding hiervoor

eerdere observaties met soortgelijke vaccinkandidaten waarbij Env specifieke antilichamen betrokken leken te zijn bij deze verhoogde gevoeligheid. In experimenten waarbij plasma van gevaccineerde katten werd toegediend aan naïeve katten was aangetoond dat deze naïeve katten vervolgens gevoeliger waren voor FIV infectie. Om uit te zoeken of specifieke gebieden in Env hiervoor verantwoordelijk waren werden Env preparaten geconstrueerd waaruit die delen verwijderd waren. Het ging hierbij om de zogenaamde hypervariabele (HV) gebieden 3 – 5, gebieden waartegen katten die gevoeliger bleken voor infectie preferentieel antilichaamresponsen leken te hebben ontwikkeld.

De virusreplicatiekinetiek in katten gevaccineerd met de originele Env preparaten (FIV-Env-ISCOM) en in katten gevaccineerd met de deletieconstructen (FIV-ΔEnv-ISCOM) bleek echter vrijwel identiek, hetgeen suggereert dat antilichamen tegen HV3-5 niet alleen verantwoordelijk zijn voor de eerder gevonden verhoogde gevoeligheid voor infectie. In tegenstelling tot de katten gevaccineerd met FIV-Env-ISCOMs vond versnelde infectie in de katten gevaccineerd met FIV-ΔEnv-ISCOMs plaats terwijl er géén virus neutraliserende antilichamen konden worden aangetoond in het CrFK systeem. De FIV-ΔEnv-ISCOM preparaten waren desondanks wél immunogeen en neutralisatie in het CrFK systeem was in deze katten na infectie eerder meetbaar dan in niet gevaccineerde dieren. Dit suggereerde dat de FIV-ΔEnv-ISCOMs een priming van het immuunsysteem bewerkstelligden. Mogelijk waren deze virus neutraliserende antilichamen die *in vitro* konden worden gemeten, verantwoordelijk voor een versnelde infectie *in vivo*. Deze hypothese zou verder dienen te worden getest. Nadere analyse van de antilichaamresponsen suggereerde tevens dat antilichamen gericht tegen andere HV gebieden dan HV3-5 betrokken waren bij de versnelde infectie in de katten gevaccineerd met FIV-ΔEnv-ISCOM preparaten. Concluderend kan gesteld worden dat verwijdering van hypervariabele gebieden uit de envelop waarschijnlijk niet de juiste strategie is om de werking van FIV vaccins en wellicht zelfs lentivirale vaccins in het algemeen te verbeteren. Ook is duidelijk dat de rol van nadelige antilichaamresponsen in verhoging van de gevoeligheid van de gastheer voor lentivirale infectie niet zo eenduidig is als in eerste instantie door verschillende onderzoeksgroepen werd gesuggereerd.

In andere studies is gesuggereerd dat algemene activatie van het immuunsysteem ook een rol zou spelen in vaccin gemediëerde versneld optredende infectie. Om deze hypothese te testen werd een aantal gevaccineerde katten in het huidige experiment niet geïnfecteerd na de gebruikelijke vier weken maar pas twaalf weken na de laatste vaccinatie. Omdat de resultaten van deze infectie experimenten niet eenduidig bleken kon geen conclusie worden getrokken over de mogelijke invloed van dit mechanisme.

Gezien de weinig succesvolle resultaten bij de ontwikkeling van kandidaat FIV vaccins die primair gericht was op het opwekken van neutraliserende antilichamen, is in de hoofdstukken 4 en 5 onderzocht of vaccinstrategieën gericht op het opwekken van cellulaire afweerreacties een kansrijker alternatief vormden. De keuze van FIV eiwitten die in zulke vaccins vertegenwoordigd zouden moeten zijn werd gebaseerd op eerder onderzoek naar de specificiteit en effectiviteit van deze T cel responsen in HIV en andere lentivirale infecties. Zo werd aangetoond dat in zogenoemde "long term non-progressors", HIV-1 geïnfecteerde patiënten die virus replicatie gedurende langere tijd onder controle hebben en waarbij AIDS aanmerkelijk later optreedt dan in "fast progressors", cellulaire afweerreacties significant vaker gericht zijn tegen de kleine, regulatoire eiwitten Rev, Tat en Nef. Deze eiwitten komen relatief vroeg in de replicatiecyclus van het virus tot expressie en de hypothese is dat een sterke cellulaire afweerreactie tegen deze eiwitten daardoor het meest effectief is in het onderdrukken van de virusreplicatie. Experimenten *in vitro* waarbij gebruik werd gemaakt van het verwisselen van virale epitopen van het ene (late) naar het andere (vroege) eiwit onderbouwden deze hypothese. Vaccinatie experimenten in het simian immuundeficiëntie virus (SIV)-makaken model hebben vervolgens aangetoond dat inclusie van deze regulatoire eiwitten in SIV vaccinpreparaten daadwerkelijk significant bijdraagt aan de werkzaamheid van zulke vaccins.

In hoofdstuk 4 zijn de regulatoire FIV eiwitten Rev en OrfA, gezuiverd verkregen middels een bacterieel expressiesysteem, al dan niet in combinatie met een bacterieel tot expressie gebracht envelop preparaat, gebruikt als subunitvaccins. De gezuiverde vaccinpreparaten werden geadjuveerd met ISCOM-matrix. Echter, geen van de vaccinpreparaten bleek in staat om afweerreacties op te wekken die beschermden tegen FIV infectie. Hoewel antilichaam responsen tegen de verschillende FIV eiwitten meetbaar waren na vaccinatie, konden geen vaccin gemediëerde, specifieke cellulaire afweerreacties aangetoond worden middels een zogenoemde interferon-γ ELISPOT test. Na infectie waren deze reacties wel meetbaar in een groot deel van de katten maar er bleken geen verschillen meetbaar tussen de verschillende vaccingroepen en tevens de controlegroepen die niet met FIV eiwitten gevaccineerd waren. De gebruikte vaccins zijn blijkbaar niet in staat om deze reacties voldoende efficiënt in de katten op te wekken. Een andere verklaring zou kunnen zijn dat in Rev en OrfA door hun geringe grootte slechts een beperkt aantal mogelijke T cel epitopen voorkomen waardoor de kans op een specifieke afweerreactie relatief klein is. Andere groepen hebben aangetoond dat het aantal reactieve T cel epitopen in deze eiwitten inderdaad beperkt is. Ook is wel gesuggereerd dat T celreacties tegen deze eiwitten zeer tijdelijk van karakter zijn waardoor hun efficiëntie beperkt en alleen meetbaar zou zijn kort na vaccinatie.

Samenvattend kan worden gesteld dat het gebruik van FIV regulatoire eiwitten Rev en OrfA, individueel of in combinatie met Env preparaten, in subunit vaccins niet resulteerde in het opwekken van beschermende afweerreacties.

Het is niet ondenkbaar dat het aanbieden van deze regulatoire eiwitten in een andere vaccinformulering, die wel in staat is om significante cellulaire afweerreacties op te wekken, bescherming tegen infectie zou kunnen bewerkstelligen. Hiertoe is in hoofdstuk **5** gebruik gemaakt van een tweetal vectorsystemen om Rev en OrfA te presenteren aan het immuunsysteem. Eerst werden katten gevaccineerd met Semliki Forest Virus (SFV) DNA vectoren waarna een boostervaccinatie werd uitgevoerd met recombinant modified Vaccinia virus Ankara (MVA) preparaten, die beide geconstrueerd waren om FIV Rev en OrfA tot expressie te brengen. Het gebruikte, zogenaamde prime-boost, vaccinatieschema heeft zich bewezen in meerdere (lenti)virale vaccinatie/infectie modellen.

Hoewel vector-specifieke antilichaamresponsen meetbaar waren na vaccinatie, konden Rev en OrfA-specifieke afweerreacties niet aangetoond worden. Dit was zowel het geval voor specifieke antilichamen als voor specifieke cellulaire afweerreacties. Het is dan ook niet verwonderlijk dat geen van de aldus gevaccineerde katten beschermd was tegen infectie met FIV. Als meest waarschijnlijke oorzaak voor het afwezig blijven van de beoogde specifieke afweerreactie werd inefficiënte expressie van Rev en OrfA door de respectievelijke vectorpreparaten genoemd. De hier beschreven experimenten laten zien dat hoewel werkzaam in andere lentivirale vaccinmodellen, de SFV en MVA presentatie systemen goed werkzaam waren voor het opwekken van antivirale afweerreacties tegen Rev, Tat en Nef, de door ons gebruikte systemen niet geschikt zijn voor het opwekken van afweerreacties tegen Rev en OrfA die katten zouden kunnen beschermen tegen infectie met FIV.

Voor de ontwikkeling van een FIV vaccin is het ook belangrijk om te weten hoe groot de genetische variatie van het virus is, zowel op populatieniveau als in individuele, FIV geïnfecteerde katten. Hoewel er veel gepubliceerd is over de genetische variatie in kattenpopulaties wereldwijd is er nog relatief weinig bekend over de genetische variatie die optreedt gedurende FIV infectie in individuele katten. Hiertoe is in hoofdstuk **6** bestudeerd hoe deze variatie zich ontwikkelt in experimenteel geïnfecteerde katten over een periode van enkele maanden tot 3 jaar na infectie. Katten werden geïnfecteerd met een biologisch isolaat (AM19), dan wel een moleculair kloon verkregen uit dezelfde kat (19k1). De reden voor het gebruik van deze twee inocula is dat er in het eerste geval onderzocht kon worden hoe de genetische variatie in een initieel gemengde populatie virussen zich over de jaren ontwikkelt terwijl in het tweede geval onderzocht kon worden hoe deze variatie

zich ontwikkelt wanneer een kat geïnfecteerd wordt met een gekloneerd virus. Voor sequentieanalyse is gekozen voor de virale genen Env, Rev en OrfA. De laatste twee zijn gekozen omdat deze eiwitten doelwit zijn van cellulaire afweerreacties terwijl tegen Env eveneens virus neutraliserende) antilichamen worden opgewekt. Voor HIV-1 infectie bij de mens is aangetoond dat vooral virus neutraliserende antilichamen betrokken zijn bij de evolutie van de genetische variatie van envelop sequenties

Allereerst werd de genetische variatie in de FIV AM19 en 19k1 stocks die gebruikt werden voor infectie geanalyseerd. Hiermee is vast te stellen of de genetische variatie in de katten op verschillende tijdstippen na infectie het resultaat is van nieuw in de dieren ontstane varianten dan wel dat deze reeds aanwezig waren in de voor infectie gebruikte virus stocks. Zoals verwacht was de mate van variatie het grootst in de virus stock van het biologische isolaat, AM19. Geen enkele gevonden envelopsequentie van het externe deel van het eiwit was identiek. In de 19k1 virusstock waren, zoals eveneens verwacht, de meeste virussequenties daarentegen identiek.

De mate van genetische variatie gedurende de gehele periode na infectie bleek zeer beperkt te zijn voor zowel de AM19 als de 19k1 geïnfecteerde katten. Hoewel er meer varianten in de AM19 geïnfecteerde katten gevonden werden bleken deze vrijwel allemaal reeds aanwezig te zijn geweest in de virus stock die gebruikt werd voor infectie en er bleek dus nauwelijks sprake van het ontstaan van nieuwe genetische varianten. In tegenstelling tot de situatie bij HIV-1 infectie kon er dus geen bewijs gevonden worden voor evolutie van genetische variatie onder invloed van virus neutraliserende antilichamen.

In de kat geïnfecteerd met 19k1 die over een periode van bijna 3 jaar gevolgd kon worden werden meerdere mutaties gevonden in zowel Env als OrfA in het laatste genomen monster op 143 weken na infectie. Deze mutaties vielen samen met klinische observaties die erop duidden dat het ziektebeeld bij deze kat zich progressief richting AIDS ontwikkelde. Dit zou erop kunnen wijzen dat de studieperiode van 3 jaar wellicht te kort is geweest voor het optreden van significante mutaties in de geïnfecteerde dieren. Desalniettemin staat de zeer beperkte hoeveelheid gevonden mutaties in contrast met de situatie zoals die zich in HIV-1 geïnfecteerde mensen voordoet, al dient gezegd dat de genetische variatie tussen geïnfecteerde individuen sterk kan variëren. In dit opzicht was het opvallend dat het reverse transcriptaseeiwit (RT) dat bij de virusreplicatie betrokken is en waarvan de beperkte betrouwbaarheid verantwoordelijk is voor de introductie van foutieve basenparen in de gebruikte FIV stammen een genetische samenstelling heeft die overeenkomt met meer stabiele vormen van het eiwit zoals die in studies naar de betrouwbaarheid van SIV RT beschreven is. Mogelijk heeft FIV RT van zichzelf een hogere betrouwbaarheid dan die van de bestudeerde RT enzymen van andere lentivirussen. Een andere verklaring voor het gebrek aan genetische variatie zou gevonden kunnen worden in het feit dat

FIV wellicht een inherent lagere replicatiesnelheid heeft dan andere lentivirussen zoals bijvoorbeeld HIV-1. Een lagere replicatiesnelheid zoals beschreven voor HIV-2 en het bovine immuundeficiëntie virus (BIV) suggereert dat dit wellicht samenvalt met een lagere virulentie en relatieve genetische stabiliteit. Hiertegenover staat echter dat tijdens infectie van pony's met het equine infectious anemia virus (EIAV) sequentie-evolutie tijdens periodes van onderdrukte virusreplicatie niet onderdeed voor de variatie die optrad tijdens periodes van verhoogde virusreplicatie.

Samengevat kan gesteld worden dat wellicht meer genetische variatie gevonden zou kunnen worden bij gebruik van meer virulente FIV stammen en bij het langer vervolgen van de infectie dan 3 jaar. Aan de andere kant zou de gevonden beperkte genetische variatie erop kunnen duiden dat er voor bepaalde FIV stammen die endemisch zijn in bepaalde kattenpopulaties gemakkelijker vaccins voor "regionaal gebruik" ontwikkeld zouden kunnen worden.

Conclusies

Ondanks de enorme investeringen die gedaan zijn voor het ontwikkelen van lentivirale vaccins is men er tot op heden nog niet in geslaagd een effectief en veilig vaccin maken. Een uitzondering lijkt het recentelijk in de VS op de markt gebrachte vaccin tegen FIV, Fel-O-Vax™, hoewel de effectiviteit van dit vaccin, zeker tegen bepaalde heterologe FIV stammen, ter discussie staat.

Één van de grootste problemen is dat zelfs nu nog de zogenoemde "correlates of protection", d.w.z. welke (virus-specifieke) afweerreacties er precies nodig zijn voor bescherming, voor de verschillende lentivirussen nog altijd grotendeels onbekend zijn. Studies in mens en dier hebben aanwijzingen opgeleverd dat bepaalde virusneutraliserende antilichamen en T cel-responsen belangrijk zijn. Daarnaast hebben T cel depletie studies in makaken laten zien dat cellulaire responsen een rol spelen in het vertragen van het ontstaan van AIDS. Experimentele overdrachtstudies met plasma en lymfocyten hebben laten zien dat beide inderdaad een belangrijke rol kunnen spelen in vaccin gemediëerde bescherming tegen infectie. Er zijn echter veel uitgebreidere en gedetailleerde studies nodig om daadwerkelijk gebruik te kunnen maken van het opwekken van deze responsen bij het ontwikkelen van werkzame lentivirale vaccins .

Nu ook voor katten steeds meer relevante reagentia beschikbaar worden om deze analyses uit te kunnen voeren, zoals de IFN-γ ELISPOT test, kan meer geïnvesteerd worden in het ontrafelen van deze "correlates of protection". Daarnaast kan de recente

ontwikkeling van testen die niet meer afhankelijk zijn van soortspecifieke reagentia, zoals de FATT-CTL test, de identificatie van de beschermende responsen die nodig zijn voor de rationele ontwikkeling van de beoogde vaccins, vergemakkelijken.

Naast bovengenoemde voorwaarden is een andere belangrijke rol weggelegd voor het ontwikkelen van meer adequate presentatiesystemen voor vaccincomponenten. Het is dan ook belangrijk te beseffen dat hoewel de resultaten behaald met de op FIV Rev en OrfA gebaseerde kandidaatvaccins beschreven in hoofdstukken 4 en 5 suggereren dat deze eiwitten niet geschikt zouden zijn om bescherming tegen FIV infectie op te wekken, dat verbeterde presentatie en vaccinformuleringen (betere adjuvantia) de werkzaamheid van op Rev en OrfA gebaseerde vaccins zouden kunnen verbeteren.

De eerdergenoemde flexibiliteit van het FIV-kattenmodel met betrekking tot het inzetten van verschillende infectieroutes behoeft ook verdere uitwerking. Deze flexibiliteit is aantrekkelijk en een belangrijk argument voor nader onderzoek aan transmissie van lentivirussen in het algemeen en de ontwikkeling van verbeterde, meer specifiek toegespitste interventiestrategieën in het bijzonder. Daarnaast is het voor het veterinaire doel, de ontwikkeling van een veilig en effectief FIV vaccin voor katten, van belang dat er meer onderzoek wordt gedaan naar het opzetten van een infectiemodel dat meer toegespitst is op de situatie zoals die zich in werkelijkheid voordoet bij de transmissie van het virus van kat naar kat. Tot dusver zijn meestal infectiedoses gebruikt die de werkelijke overgedragen dosis waarschijnlijk vele malen overschrijden waardoor de geteste kandidaatvaccins wellicht onterecht als niet effectief beschouwd worden. Het is daarom wenselijk vanuit het oogpunt van de ontwikkeling van veterinaire FIV vaccins meer natuurlijk relevante infectie modellen op te zetten.

Er zijn helaas vele voorbeelden van niet succesvolle lentivirale vaccinkandidaten. Voorts zijn er zelfs vaccins beschreven die leiden tot een grotere gevoeligheid voor infectie in gevaccineerde dieren (zie hoofdstuk 7). En hoewel niet aangetoond is dat deze verhoogde gevoeligheid een specifiek verschijnsel voor katten is zou een verbeterd inzicht in de mechanismen die betrokken zijn bij het ontstaan van dit fenomeen van belang kunnen zijn voor vaccinontwikkeling in het algemeen en die van lentivirale vaccins in het bijzonder. Het resultaat van vaccinatie-infectie experimenten zou wellicht als de som gezien kunnen worden van een tweetal door vaccinatie bewerkstelligde processen: bescherming en verhoogde gevoeligheid voor infectie.

Met de recente identificatie van de T cel activatiemarker CD134 als de primaire receptor voor FIV is er een curieuze paradox bij de ontwikkeling van FIV vaccins ontstaan – de cellen die nodig zijn voor het opwekken en aanhouden van door vaccinatie gemediëerde afweerreacties zijn niet alleen de primaire doelwitten van het virus, hun functie als dirigent van de afweerreacties van de gastheer kan resulteren in het tegenovergestelde effect: het vergemakkelijken van de verspreiding van het virus tijdens acute infectie. Hoewel het verantwoordelijke mechanisme anders is dan dat bij HIV infectie, zouden experimenten gericht op het bestuderen van vaccinatie gemediëerde stimulatie van deze cellen antwoorden kunnen opleveren die van belang zijn voor de ontwikkeling van vaccins tegen zowel FIV als HIV. Deze experimenten zouden zich niet moeten beperken tot het gebruik van FIV vaccins alleen, ook het gebruik van vaccins tegen ongerelateerde ziektekiemen zou wellicht kunnen resulteren in een verhoogde gevoeligheid voor FIV infectie. Ook het gebruik van lentivirale vaccins op basis van virale of bacteriële vectoren waartegen de te vaccineren individuen reeds immuniteit hebben opgebouwd dient met de nodige voorzichtigheid te worden betracht.

Afsluitend kan worden geconcludeerd dat, hoewel er fundamentele verschillen bestaan tussen de pathogenese van FIV infectie in katten enerzijds en HIV infectie in mensen anderzijds, de overeenkomsten waarschijnlijk een grotere relevantie hebben met betrekking tot lentivirale vaccinontwikkeling. Vaccinontwikkelaars voor beide infecties zien zich nog altijd geconfronteerd met uitdagingen die voor een groot deel dezelfde zijn. Daarom is het ook gezien de voordelen die het FIV-kattenmodel voor lentivirusonderzoek biedt, belangrijk dat onderzoek naar zowel de pathogenese van de infectie als de ontwikkeling van vaccins ertegen onverminderd doorgaat. Ook onderzoek gericht op de identificatie van beschermende afweerreacties en wellicht minstens zo belangrijk op afweerreacties die verhoogde gevoeligheid voor infectie bewerkstelligen verdient onverminderde aandacht.

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Willem Huisman
31 oktober 2008.

Curriculum Vitae

Willem Huisman werd te Utrecht geboren op 4 april 1972. Na het behalen van het VWO (Atheneum) diploma aan de Aletta Jacobs Scholengemeenscap te Hoogezand werd aan het einde van de zomer van 1990 begonnen met de studie Biologie aan de Universiteit Utrecht. Op de afdeling Moleculaire Celbiologie van de Universiteit Utrecht van Prof. dr. H.O. Voorma doorliep hij zijn eerste stage onder begeleiding van dr. A.A.M. Thomas en drs. H. Teerink. Aldaar werd onderzoek verricht aan de regulatie van translatie-initiatie in eukaryote celsystemen. Zijn afstudeerstage doorliep hij in Rotterdam op de afdeling Virologie van het Erasmus MC van Prof. dr. A.D.M.E. Osterhaus. Onder begeleiding van dr. C.H.J. Siebelink werd onderzoek verricht aan epitopen in het envelopeiwit van het feline immuundeficiëntie virus (FIV) die het doelwit vormen van virus neutraliserende antilichamen. Daarnaast werd de effectiviteit van FIV vaccins getest in vaccinatiestudies. Na het behalen van het Biologie diploma in 1997 werd in datzelfde jaar begonnen aan een promotieonderzoek op de afdeling Virologie van het Erasmus MC, onder begeleiding van Prof. dr. A.D.M.E. Osterhaus en dr. G.F. Rimmelzwaan. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Sinds oktober 2006 is hij werkzaam als projectleider op de afdeling Virologische R&D van Nobilon International BV alwaar in samenwerking met het Nederlands Vaccin Instituut wordt gewerkt aan de ontwikkeling van een vaccin tegen het respiratoir syncytieel virus (RSV).

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