Neutralisierende Antikörper gegen die
transmembranen Hüllproteine von Retroviren

Dissertation

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Disputation am

Table of contents

Abbreviations

aa amino acid
AIDS Acquired Immune Deficiency Syndrom
Amp ampicillin
CBP calmodulin-binding protein
CCR chemokine receptor for CC chemokines
CD cluster of differentiation
CHR C-terminal helix region
CTL cytotoxic T-cells
CXCR chemokine receptor for CXC-chemokines
ddH2O Aqua bidest
DNA Desoxyribonucleinacid
E.coli Escherichia coli
ELISA enzyme linked immunosorbent assay
Env envelope protein
Fab fragment antigen binding
FeLV feline leukemia virus
FIV Feline Immune Deficiency Virus
FCS fetal calf serum
ffu/ml focus forming units per milliliter
FP fusion peptide
Gag group specific antigen
HAART highly active anti retroviral therapy
HAT hypoxanthine-aminopterin-thymine
HIV human immune deficiency virus
HTLV human T cell leukemia virus
Ig immunglobuline
IN Integrase
k$_A$ association constant
k$_D$ dissociation constant
kB kilo base
kD kilo Dalton
LTR long terminal repeat
MCS multiple cloning site
MHC major histocompatibility complex
MVA modified vaccinia Ankara virus
Nef negative factor
NHR N-terminal helix region
NIAID National Institute of Allergy and Infectious Diseases
NIH National Institute of Health
NMR nuclear magentic resonance,
PBS phosphate buffered saline

PCR polymerase chain reaction
PEG poly ethylene glycol
PERV Porcine Endogenous Retrovirus
Pol Polymerase
POD Peroxydase
RNA Ribonucleinacid
RT Reverse transcriptase
SCID severe combined immune deficiency
SHIV SIV-HIV-hybride viruses
SIV Simian Immune Deficiency Virus
Tat Transactivator of transcription
TM transmembrane region
UNAIDS United Nations Department of AIDS
Vif virion infectivity factor
WHO World Health Organisation
wt Wildtyp

Amino acid sequences in single letter code:
A Alanin
C Cystein
D Asparaginacid
E Glutaminacid
F Phenylalanin
G Glycin
H Histidin
I Isoleucin
K Lysin
L Leucin
M Methionin
N Asparagin
P Prolin
Q Glutamin
R Arginin
S Serin
T Threonin
V Valin
W Tryptophan
X optional amino acid
Y Tyrosin

Bases of nucleotide sequences:
A Adenin
C Cytosin
G Guanin
T Thymin

1. Introduction

1.1 Retroviruses

The family of *Retroviridae* is divided into seven genera (Table 1). The virus-host interaction of most retroviruses is restricted to mammalians causing a variety of symptoms including oncological, neurological and immunodeficient deseases as well as inapparent courses of infection. Retroviruses have a single stranded RNA genome and a double stranded DNA genome serving as an intermediate product during virus replication and integration into the host cell genome. In 1970 H.M. Temin, S. Mituzami and D.Baltimore discovered the retroviral enzyme reverse transcriptase that allows the transcription of single stranded RNA into double stranded DNA. In 1980 Robert C. Gallo described for the first time a retrovirus inducing T-cell leukaemia in humans (HTLV) and shortly after the human immunodeficiency viruses HIV-1 and HIV-2 causing the aquired immune deficiency syndrom (AIDS) in infected humans (for review see Karpas 2004).

Table 1 Retrovirus genera. Adapted from Overbaugh et al., 2001

Genus	Morphology	Examples[a]
Alpharetrovirus	C type	RSV, ASLV
Betaretrovirus	B and D type	MMTV, SRV-1 to SRV-5, BaEV, JSRV, ENTV
Gammaretrovirus	C type	MoMLV, A-MLV, 10A1 MLV, X-MLV, P-MLV, AKV, GALV, MDEV, FeLV, PERV, RD-114, SNV, REV
Deltaretrovirus		HTLV-1, HTLV-2, STLV-1 to STLV-3, BLV
Epsilonretrovirus		WDSV
Lentivirus		HIV-1, HIV-2, SIV
Spumavirus		HFV, SFV

a RSV, Rous sarcoma virus; MMTV, mouse mammary tumor virus; ENTV, enzootic nasal tumor virus; MoMLV, Moloney MLV; X;MLV, xenotropic MLV; P-MLV, polytropic MLV; AKV, AKV MLV; MDEV, M. dunni endogenous virus; REV, reticuloendotheliosis virus; STVL, simian T-lymphotropic virus; BLV, bovine leukemia virus; WDSV, walleye dermal sarcoma virus; HFV, human foamy virus; SFV, simian foamy virus.

Retroviruses exist in two different forms: (i) Exogenous viruses bear genetic information necessary for the generation of replication competent viral particles and are able to be transferred from one organism to another. (ii) Endogenous viruses are vertically transmitted through the germline of its host and most of them have lost the genetic information to produce viral particles. For example retrotransposons are present in about one percent of the human genome, only existing of the genome flanking sequences of ancient retroviruses. However, under certain circumstances the function of endogenous viruses can be restored, e.g. by the infection of an exogenous virus showing a certain degree of homology with the endogenous one. For example subgroup B FeLVs evolve by recombination with portions of endogenous FeLV-like envelope sequences, which have a high degree (~80%) of homology to FeLV-A (Anderson et al., 2001).

All infectious retroviral particles show a similar structure with a diameter of about 100nm. The viral membrane is built up mainly by its host cell cytoplasm membrane, associated with the viral envelope proteins.

The matrix proteins (MA) are associated with the viral membrane by amino terminal myristyl acids forming an isometric structure of the particles. The virus capsid is encased by the viral membrane and contains the group specific antigen proteins (Gag). It contains two unattached copies of the single stranded RNA virus genome, which is associated with nucleocapsid proteins (NC). The capsid membrane is connected to the capsid core by the p6 Link protein (in HIV-1) and contains also the enzymatic reactive gag proteins reverse transcriptase, integrase and protease.

The retroviral genome is differing in sizes from 7000 to 12000 base pairs depending on the type of virus (HIV: 9000bp, FeLV-A 8500bp), encoded by a single stranded RNA consisting of a 5`cap structure and a 3´ polyadenylation signal. Retroviral genomes encode for proteins Gag (group specific antigens), Pol (enzymatic activities) and Env (envelope glycoproteins). More complex retroviruses such as lentiviruses, spumaviruses and HTLV encode additional regulatory and accessory proteins. The coding regions are flanked by regulatory control sequences termed long terminal repeats (LTR) (Fig.1A). These sequences of repetitions contain three different regions termed U3, R and U5 which are located at the 3´end and the 5´end in the provirus genome at the same orientation. The LTR bears all *cis* active sequences as well as elements of the promoter and enhancer sequences controlling the gene expression. In addition the LTR´s are essential for the process of reverse transcription and integration of the provirus DNA into the host cell genome. Cellular proteins transactivate the transcription of viral protein sequences mainly by binding to the U3 region. For example the nuclear factor kappa B (NFκB) and cytokines IL-1 or TNF-α bind to their specific sequence within the HIV-1 U3 region and activates viral transcription. This mechanism plays an important role in the pathogenic course of an HIV infection due to the fact that any stimulation of the immune system resulting in an increasing activity of NFκB may lead to the expression of HIV in its host.
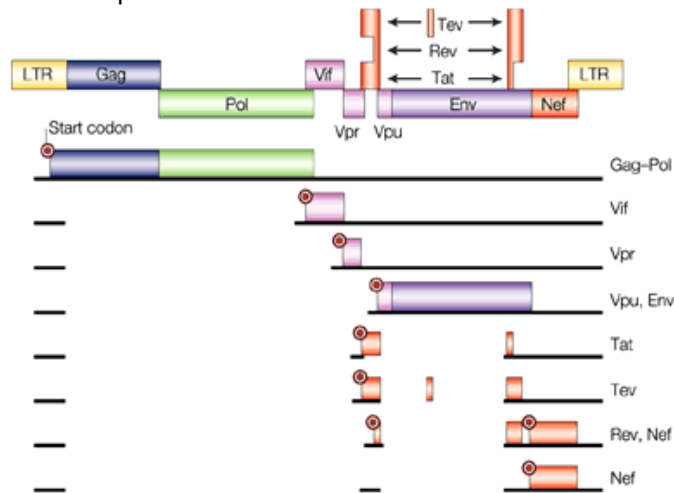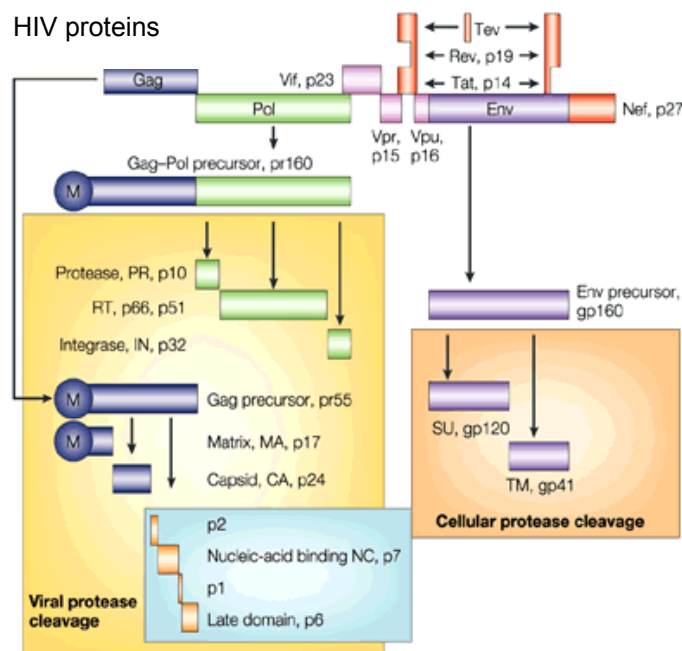
Figure 1 The HIV genome, transcripts and proteins. (**A**) HIV transcripts. Integrated into the host chromosome, the 10-kb viral genome contains open reading frames for 16 proteins that are synthesized from at least ten transcripts. Black lines denote unspliced and spliced transcripts, above which coding sequences are given, with the start codons indicated. Of these transcripts, all singly spliced and unspliced transcripts shown above those encoding the transcriptional transactivator (Tat) require regulator of virion gene expression (Rev) for their export from the nucleus to the cytoplasm. The RNA target for Rev, the Rev response element (RRE), is contained in the gene encoding envelope protein (Env). (**B**) HIV proteins. Group-specific antigen (Gag) and Gag–Pol (polymerase) polyprotein precursors are processed by the viral protease into nine subunits: protease (PR), reverse transcriptase (RT), which contains RNAse H, integrase (IN), matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1 and p6 (shown in the yellow box). Env is cleaved by cellular proteases, such as furin, into surface (SU) gp120 and transmembrane (TM) gp41 moieties (shown in the orange box). Tat is the main transcriptional regulator of the long terminal repeat (LTR). Its RNA target, the transactivation response (TAR) element, is present at the 5' end of all viral transcripts. Rev is the main nuclear-export protein and it regulates the shift between early and late viral gene expression. The viral-infectivity factor (Vif), viral protein r (Vpr), viral protein u (Vpu) and negative effector (Nef) proteins are known as accessory proteins because they are dispensable for viral growth in some cell-culture systems. Nevertheless, they have essential roles in viral replication and progression to AIDS *in vivo*. Arrows below polyprotein precursors point in the direction of their processing to mature proteins. Tev contains Tat, Env and Rev sequences and functions as Tat and Rev. Adapted from Peterlin & Trono 2003.
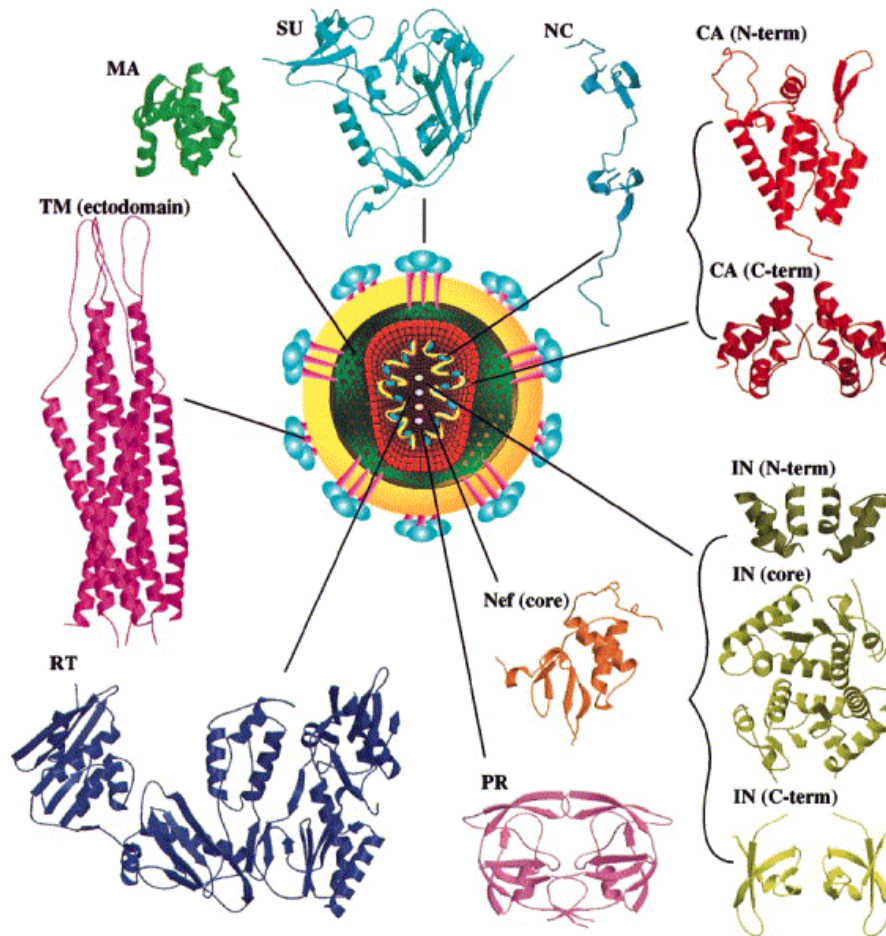
Figure 2 Drawing of the mature HIV virion surrounded by ribbon representations of the structurally characterised viral proteins and protein fragments. The protein structures have been drawn to the same scale. The TM ectodomain shown is that determined for the closely related SIV. Adapted from Turner and Summers 1999.

1.1.1 Gag (group specific antigens)

The retroviral *gag* gene encodes for a precursor protein with a size of 55kDa in case of HIV and 65kDa in case of FeLV, synthesized on ribosomes in the cytoplasm of the host cell. During maturation of the virus the protein is being processed by the viral protease into the matrix protein (MA), the capsid protein (CA), the nucleocapsid protein (NC) and in case of HIV also into the link protein (p6). The sequential position of the gag proteins located in the retroviral genome (Fig.1) is equal for all retroviruses. The gag precursor proteins are essential for particular structures during virus morphogenesis. The nucleocapsid protein forms together with the viral RNA a ribonucleo-protein complex by the interaction with a leader sequence termed ψ- region on the RNA genome. By this mechanism it supports the assembly of the RNA genome and of the t^{Lys}-RNA primer in newly generated viral particles (De Rocguigny et al., 1992; Gorelik et al., 1993) and initiates the reverse transcription. In HIV this protein-nucleotide interaction is mediated by motifs similar to zinc finger

domains. The matrix proteins aggregate in trimeric complexes and are associated by their N-terminal myristylations with the interior of the viral membrane. It mediates the transport of the DNA provirus genome to the cellular nucleus. Unlike all other retroviruses, in case of HIV the matrix protein allows the infection of non-proliferating host cells. In HIV the p6 link protein interacts with the vacuolar protein sorting (Vps) machinery (Strack et al., 2003) and is essential for the budding of the virus from the cellular membrane.

1.1.2 Pol (enzymatic activities)

Genes encoding for the viral protease, the reverse transcriptase and the integrase (*pol* genes) are processed as a single precursor protein together with the gag proteins located N-terminal. This fusion protein has a size of 160 kDa in HIV-1 and 140kDa in FeLV-A. Its synthesis is mediated by a ribosomal frame shift within a uridine rich region of the mRNA also serving for the translation of the gag proteins. Due to a hairpin conformation of the mRNA protein synthesis is slowed down and an occasional frame shift (of -1 in HIV-1) leads to the expression of the precursor protein in about five percent of translations. The gag/pol precursors are myristilated at their N-terminus and are processed sequentially into their enzymatic components by the protease during the maturation of the virus.

The protease is similar to aspartyl proteases and is organised as a homodimer with a molecular weight of 9 to 10kDa. The enzymatic center consists of two asparagin acids essential for its enzymatic activity.

The HIV-1 reverse transcriptase (RT) is magnesium (Mg^{2+}) dependant and catalyzes the transcription of viral RNA into proviral DNA. In addition to that it bears an RNase H activity allowing degradation of RNA from DNA-RNA hybrid strands. It is arranged as a heterodimer with molecular masses of 66kDa and 51kDa. The enzyme does not have a proof reading function. Thus it increases the probability of wrong base pair incorporation to 10^{-3} to 10^{-4} into newly transcribed DNA.

The integrase is part of the preintegration complex and cleaves the 5′ and the 3′LTR of the double stranded proviral DNA at their 3′ends. By the resulting ends the proviral DNA is integrated into the host cell genome followed by padding the free 3′ends of cellular DNA.

1.1.3 Env (envelope glycoproteins)

In general the surface envelope proteins mediate the absorption of the viral particle to their cellular receptor and therefore are the primary determinants of the viral host range. The interaction of the SU portion of the retroviral envelope to its receptor induces a conformational change that exposes a viral fusion peptide, present in the ectodomain of TM, allowing the viral membrane to fuse with the host cell membrane. For most retroviruses, fusion occurs at neutral pH. The process of fusion is not energetically advantageous and requires bypassing of steric and electrostatic barriers (Patel, M. et al.,

1993). Until today the mechanism of membrane fusion is not resolved completely and different fusion models exist (Fig.3).

The Env membrane precursor protein is translated from one mRNA spliced once with a size of 160kDa in HIV-1 and 85kDa in FeLV-A. An amino terminal leader sequence directs the precursor mRNA towards the endoplasmatic reticulum (ER) and chaperones mediate the translation of the protein into the lumen of the ER. The hydrophobic transmembrane domain in the TM protein anchors the precursor protein in the ER membrane (Fig.4). The proteins are arranged in trimeric complexes organised in six helix bundles. Three surface envelope proteins and three transmembrane envelope proteins form the so called knob. During the transport of Golgi vesicles from the ER towards the cellular membrane, the precursor protein becomes glycosylated and is finally cleaved by a cellular protease into the transmembrane and the surface protein component. The HIV transmembrane envelope protein gp41, as well as the surface protein gp120 contain glycosylation motifs. In FeLV only the surface envelope protein gp70 is glycosylated while the transmembrane envelope protein p15E is not.
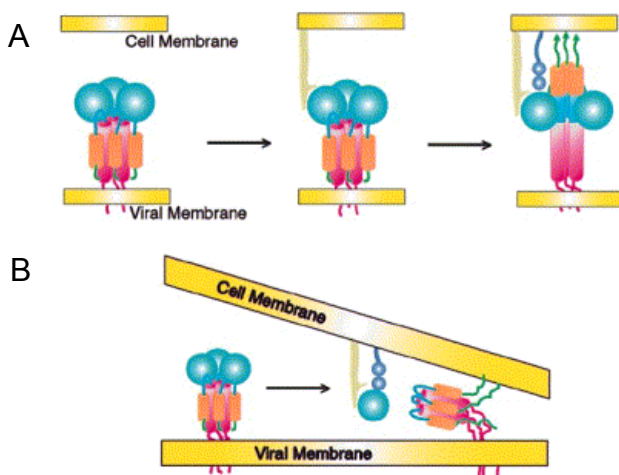
Figure 3 Potential mechanisms for CD4 and chemokine receptor induced fusion of the viral and cellular membranes. (**A**) Spring-loaded mechanism similar to that proposed for hemagglutinin, where conformational changes in the TM ectodomain lead to a major displacement of the N-terminal fusogenic peptide toward the cellular membrane.
(**B**) Shedding mechanism, where CD4 and chemokine binding result in the loss of SU proteins, enabling reorientation of the TM and membrane fusion.
Adapted from Turner and Summers 1999.

The surface envelope protein of HIV gp120 is highly glycosylated and is divided into an outer and an inner domain, connected by a β-bridging sheet. The inner domain consists of two α helices, a five stranded β-sheet and several loops. The outer domain has a double barrel structure, while one of them is built up from a six stranded β-sheet and one α-helix and the other one is organised from a seven stranded β-sheet orientated in an anti parallel form. The protein contains five highly variable regions termed V1-V5 stabilised by disulfide bindings and six conserved regions termed C1-C6. Within the variable regions amino acid deletions, insertions and changes of glycosylation sites occur during the course of infection in different isolates from a single patient. During the attachment process HIV gp120 mediates the contact to the host cell by binding the CD4 receptor followed by an interaction

with a secondary cellular co-receptor. The binding of CD4 by gp120 induces a change of conformation within the trimeric envelope protein structures (Salzwedel and Berger, 2000) and leads to the exposition of the viral co-receptor binding site (Kwong et al., 1998). The co-receptor binding is essential for the infection of a CD4$^+$ host cell (McDoughal et al., 1986) and permits either the infection of T-cells by binding to CXCR4 (Feng et al., 1996) or of monocytes and macrophages by binding to CCR5 (Alkhatib et al., 1996). Both co-receptors belong to the family of seven helix chemokine receptors. Ongoing conformational changes finally induce the loosing of gp120 from the trimeric complexes (shedding) and to the exposition of the gp41 protein towards the cellular membrane (Fig 3B).

The transmembrane enevelope protein has a size of 41kDa in HIV-1 and a size of 15kDa in FeLV-A. The extracellular components of the TM proteins within the family of retroviruses show structural homologies (Gallaher et al., 1989; Benit et al., 2001). In general retroviral transmembrane proteins are organised in three domains. From the N-terminus towards the C-terminus, the extraviral domain (ectodomain) is followed by the transmembrane domain and the cytoplasmatic part of the protein. The ectodomain is subdivided into an N-terminal fusion peptide, an N-terminal helix region (NHR) including the immunosuppressive domain (Denner et al., 1994) and a cysteine-loop connecting the N-terminal part with the C-terminal helix region (CHR). The fusion peptide consists of approximately 20 hydrophobic amino acids (Fig.4). During the process of attachment of a viral particle and its host cell, it fixes the viral membrane with the host cell membrane and thus initiates membrane fusion. Retroviral transmembrane proteins show a higher degree of conserved amino acid sequences than the surface proteins.
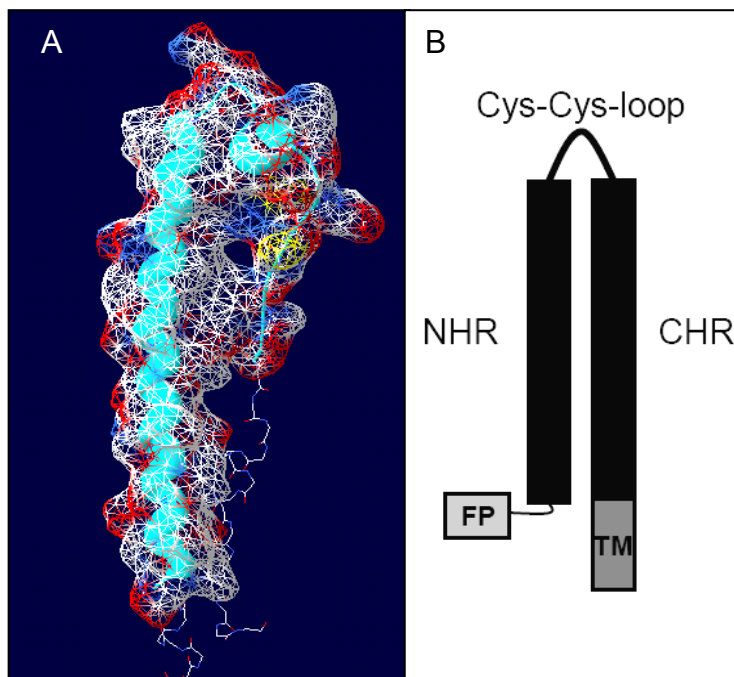
Figure 4 Models of monomeric retroviral transmembrane proteins (TM). (**A**) Theoretical model of FeLV-A p15E ectodomain generated by using Swiss model database (aa 467-583).
(**B**) Schematic model of FeLV-A p15E ectodomain inclusive the transmembrane domain. Indicated are the cysteine-loop (Cys-Cys-loop), the N- and C-terminal helix regions (NHR, CHR), the fusion peptide (FP) and the transmembrane domain (TM).

In HIV six helix bundles of the gp41 proteins are arranged by coiled coil structures of N-terminal helices stabilised by the interaction of hydrophobic conserved amino acids (Weissendorn et al., 1997). Similar molecular structures are found for gammaretroviruses (Fig.5). In contrast to other retroviruses HIV gp41 consists of a greater intraviral portion shown to play an important role in virus replication (Dubay et al., 1992, Freed and Martin, 1995).
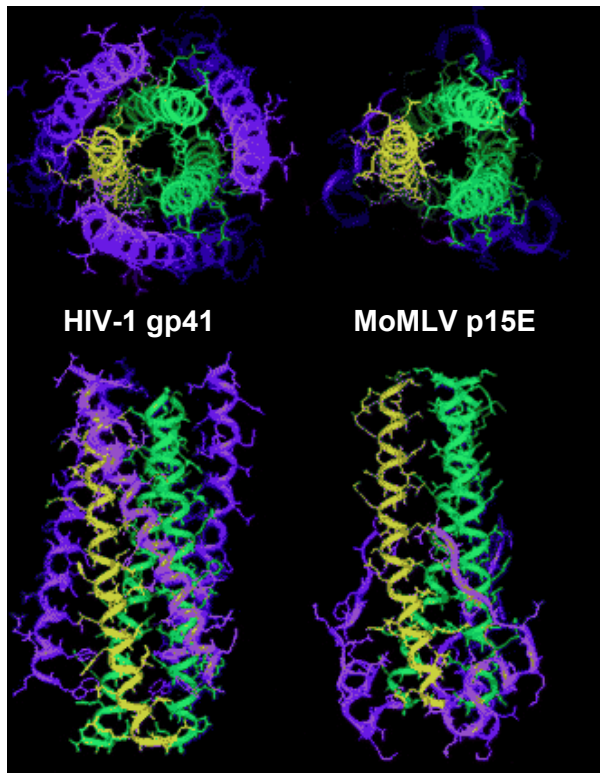
Figure 5 Comparison of HIV-1 gp41 with the gammaretroviral Mo-MLV p15E structures. The top panel shows an end-on view of the two structures from the top. The bottom panel shows a side view. The three monomers forming the central coiled coil of each structure are colored yellow, green, and blue. Supporting structures are colored purple. The figure was generated using the program Insight (Biosym). Adapted from Chan 1997.

1.1.4 Regulatory proteins

Until today the regulatory Tat protein was described for lentiviruses only. However, i.e. for HTLV and for HSRV proteins with functional regulation of transcription are also known. In the early phase of the HIV-1 infection cycle transcription of proviral DNA is incomplete due to the suppression of the elongation. In later stages of the infection cycle the Tat protein accumulates and binds to the TAR (trans-activating response) element on the newly synthesized mRNA. Thus the resulting tat-cyclin T complex can activate the cyclin-dependant protein kinase 9 (Cdk9) which leads to the phosphorylation of the RNA-polymerase II and finally to the stabilisation of the transcriptional elongation.

A post transcriptional transactivator was firstly discovered for HIV-1. This protein is essential for the time dependant regulation of gene expression during the replication cycle. In case of HIV-1 it is the Rev protein that participates in the transport mechanism of viral mRNA from the nucleus to the cytoplasm. Therefore it binds to the Rev-response element (RRE) located on the mRNA encoding for the env gene and introduces the nucleus shuttle protein exportin-1 and the nucleus factor Ran-

Guanosine-Triphosphate-GTP. By these mechanisms Rev serves as a regulator separating the transcription of early genes, which depend on mRNA spliced intensively (i.e. Tat, Rev and Nef), from the transcription of late genes (Gag, Pol, Env or accessory proteins) which depend on mRNA unspliced or spliced only once.

1.1.5 Accesory proteins

The majority of accessory proteins has been described for more complex retroviruses such as lentiviruses. In HIV-1 at least five different accessory proteins are known with distinct impacts on pathogenicity, evasion from the host immune response and influence on viral replication.

The Nef protein (negative factor protein) is an early phase protein that accumulates in the cytosol of the infected host cell. It increases viral replication and at the same time it decreases expression of CD4 molecules on the host cell membrane by clathrine mediated endocytosis. Thus CD4-Env complexes at the cell surface are prevented and the probability of a secondary HIV-1 infection of that particular host cell is reduced. Further more Nef downregulates the major histo-compatibility complex II (MHC-II) presentation on infected cells and interacts with a variety of different cellular proteins.

The Vif (viral infectiosity factor) is encoded in a reading frame between the *pol* and the *env* gene. It was supposed that the protein participates in the uncoating of the virus and in the initiation of the reverse transcription. It is assumed by that Vif mediates the proteasome depending degradation of a cellular deoxycytidine deaminase (APOBEC3G) and thus allows the production of infectious viral particles in non-permissive cells (Mehle et al., 2003).

The Vpr (virion associated protein r) binds to the nucleus import complex composed of nucleoporin and importin-α and by this it participates in the transport of the preintegration complex towards the nucleus. In addition vpr has an influence on the host cell cycle by arresting it in the G2 phase. The Vpu (viral protein U) interacts with CD4 molecules in the ER and triggers its degradation in the ubiquitine proteasome pathway, preventing generation of CD4-env complexes in the ER.

1.1.6 The retroviral cycle of replication

The retroviral cycle of infection can be divided into several distinct phases. The early phase is marked by the recognition and adsorption of the virus to the host cell membrane mediated by membrane protein complexes. In general different cellular receptors for retroviruses are known belonging to the classes of multiple- and / or single- transmembrane proteins with a variety of functions in uninfected cells (Overbaugh et al., 2001). In case of HIV-1 the cellular receptor CD4 as well as the two possible co-recpetors CXCR4 and CCR5 are well described (Markovic et al., 2004). In case of FeLV-A the receptor still remains unidentified while the multiple membrane protein Pit2 was defined as the cellular receptor for subgroup B (Anderson et al., 2001). After fusion of virus membrane and host cell

membrane the virus enters the cell by a fusion pore followed by uncoating of viral RNA. The viral RNA genome is released into the cytoplasm of the host cell and transcribed by the reverse transcriptase into viral DNA. The viral DNA associates with components of the preintegration complex (IN, MA, RT and in case of HIV with vpr and cellular HMG-I(Y)) and is transported to the nucleus where the viral integrase promotes the integration of provirus DNA into the host cell genome. In case of HIV the late phase of the infection cycle starts with the transcription of the regulatory genes *rev* and *tat* and the accessory genes. The Tat protein stabilises the elongation of transcription of viral proteins by indirect phosphorylation of the mRNA polymerase II. In this phase the gp160 protein is translated into the ER and post translational processes are initiated i.e. glycosylation and cleavage of gp160. The resulting trimer of non-covalent bound gp120 and gp41 is transported to the cell surface by Golgi vesicles. The gag polyprotein is translated from an unspliced mRNA and attaches to the Gag/Pol fusion proteins. The virus assembly is followed by budding of the virus from the cellular membrane or as described recently into endosomes of infected cells (Kramer et al., 2005). The final maturation of the infectious particle occurs outside the host cell (Fig.6).
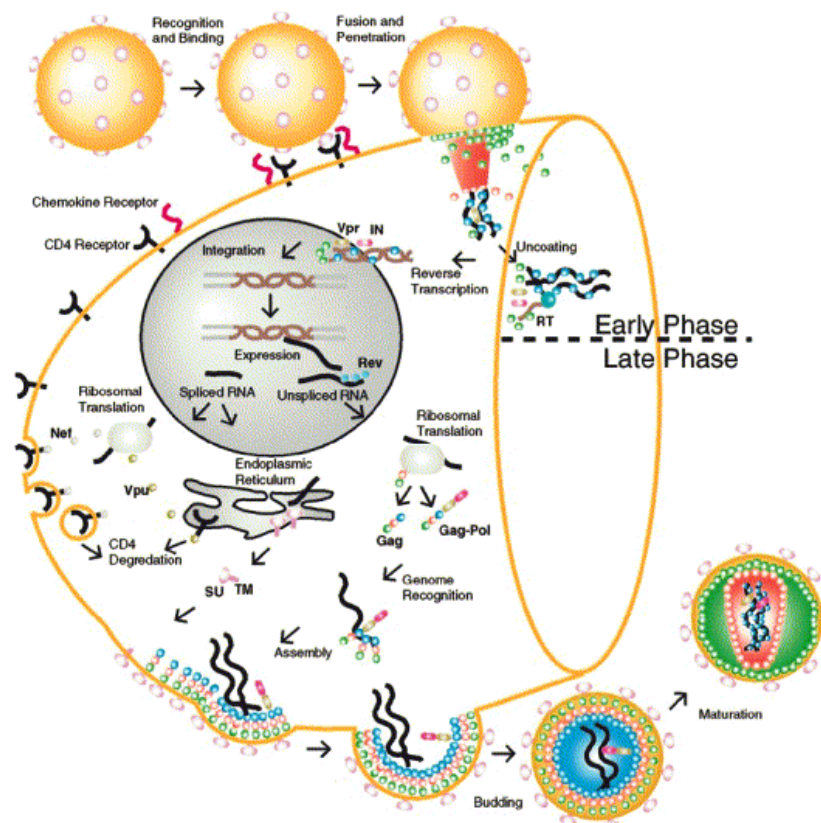
Figure 6 General features of the HIV-1 replication cycle. The early phase (upper portion of the diagram) begins with CD4 recognition and involves events up to and including integration of the proviral DNA, and the late phase includes all events from transcription of the integrated DNA to virus budding and maturation. Adapted from Turner and Summers 1999.

1.2 The interaction of the immune system with retroviral infections

1.2.1 The immune system is based on different ways of reaction specific for the type of pathogen

In general the immune response against viral infections and other pathogens can be separated in two distinct complexes of immunological reactions. The so called first line of defense is mainly based on the unspecific innate immune system. In the evolutionary progression it is the anterior system which leaded to the development of a secondary complex, the adaptive immune system (Medzhitov and Janeway, 1999). After a virus has overcome the outer barriers of the organism components of the innate immune system recognise the possible pathogen as a non-self contaminant and may be able to eliminate it. In this process monocytes, granulocytes, macrophages and natural killer cells are involved. Further more the innate immune response is supported by acute phase proteins, the complement system as well as cytokines and interferons.

The adaptive immune system is an additional response system with the capability of highly specific interactions with the pathogen. It can be subdivided into the humeral immune system consisting of antibody producing B-cells and the cellular immune system consisting of T-helper cells and cytotoxic T-cells. In a repeated infection or exposition of the pathogen the adaptive immune response is able to eliminate the contaminant faster and more effective. This adaptation is based on the clonal production of the most specific B- and T-cell response due to the mechanism of selective maturation as well as due to the generation of a specific B- and T-memory cell repertoire.

1.2.2 The innate immune system displays the first line of defense

The innate immunity uses a broad spectrum of unspecific cellular and soluble components to react on a retroviral infection. The cellular components comprise B1-cells, plasmacytoids dendritic cells (PDCs) and non-cytotoxic, antiviral CD8$^+$ cells. The soluble factors include cytokines, chemokines, α-defensines, complement, collectines, pentraxines and cathelicidines (Chang TL, et al., 2005; Levy et al., 2003). For example, the importance of the innate immunity in controlling HIV infection is becoming increasingly appreciated (Lehner 2003, Levy 2003a). The inverse correlation between the level of viremia and the ability of NK cells to inhibit HIV replication is predominantly mediated through secretion of CC chemokines, including macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES, that inhibit HIV-1 entry via CCR5 (Kottilil et al. 2003). Furthermore the PDC´s and non-cytotoxic, antiviral CD8+ have an important role in the innate immune response against HIV. PDC´s produce type1-interferones. HIV-1 positive patients with an interferon-α concentration above 300U per millilitre blood are less often affected by opportunistic infections than these which show levels below that value (Lopez et al., 1983, Siegal et al., 1986). The antiviral impact of non-cytotoxic, antiviral CD8+ cells is based on the suppression of the retroviral transcription (Copeland et al., 1995;

Levy et al., 1996). The antiviral activity of soluble factor(s) from these cells is known as CD8+ antiviral factor(s) (CAF). It is found very early in primary infection before the presence of antibodies against HIV (Mackewicz et al., 1994) and correlates with delayed disease progression in HIV-1–infected people (Walker et al., 1990; Carmichael et al., 1993; Mackewicz et al., 1991).

1.2.3 The adaptive cellular immune response is important in the defense of intra-cellular pathogens

The adaptive cellular immune response is predominantly involved into the immunological reactions against intra-cellular and phagocyted pathogens as well as into the recognition and elimination of tumor cells. The mediation of the cellular immune response is based on cytotoxic T-lymphocytes capable for the induction of apoptosis and lysis in the infected or transformed target cell. The CTL response is initiated by the MHC-I complex, presenting pathogen specific antigens on target cells.

In case of many retroviral infections, the CTL response is essential for the immunological control of the course of infection. For example in HIV infected patients it was shown that a strong CTL response correlates with a decelerated progression of infection (Rinaldo et al., 1995; Harrer et al., 1996, Greenough et al., 1997), even if a complete clearance of HIV has not been observed. In patients previously exposed to HIV-1 but not being infected a specific CTL response was shown to maintain a protection against infection to some degree (Goh et al., 1998). However, the high genetic variability of HIV-1 promotes the induction of escape mutants being resistant towards any kind of an adaptive immune response (Peeters and Sharp, 2000). In contrast to HIV-1, FeLV infected cats frequently clear circulating virus and show a correlation between clearance and the appearance of FeLV specific CTL´s (Flynn et al., 2002).

1.2.4 The B-cell mediated adaptive humoral immune response is important in the defense of extra-cellular pathogens

The B-cell mediated immune response is predominantly involved into the immunological reactions against extra-cellular pathogens and toxins. Contaminants are being bound by immunoglobulins attached to or secreted from antigen specific B-lymphocytes. Different kinds of reactions are leading either to the absorbance of the pathogen or toxin by macrophages, to its opsonisation introducing the complement system or even to its neutralisation by neutralising antibodies. In addition antibodies can recognise infected cells and initiate an antibody dependant cellular cytotoxicity (ADCC) reaction towards them. An antibody secreting B-cell specific for protein antigens previously has to be activated by an antigen specific T-helper cell (thymus-dependant reaction). In contrast B-cells secreting antibodies specific for certain ingredients of microbes, i.e. bacterial polysachharids, are not dependant on T-helper cells (thymus-independent reaction). For the effective prevention of a viral infection the

titre of neutralising immunoglobulins is of particular interest. The capability of virus neutralisation is dependant on the affinity and avidity of immunoglobulins for viral proteins critical in the mechanism of infection. In retroviruses the envelope proteins display an effective target for virus neutralisation mediated by immunoglobulins. Animals or humans infected with retroviruses show humoral immune responses against the viral envelope proteins with different capacities of virus neutralisation. However, in many cases the immunologic control of retroviral infections by these antibodies is not sufficient. For example in HIV-1 infected patients neutralising antibodies against gp120 are unable to provide virus clearance (Ruppach et al., 2000) due to high genetic variability of HIV-1 depending on the imprecisely transcribing reverse transcriptase (Preston et al., 1988, Roberts et al., 1988). In addition to that the double stranded RNA genome of HIV allows an efficient rate of recombination in host cells infected with two different subtypes. Both mechanisms of genetic variability allow HIV-1 to evade the immune response and medical therapy by the generation of escape mutants (Evans and Desroisiers, 2001; Kijak et al., 2002; Kwong et al., 2002; Wei et al., 2003).

1.2.5 Antibody characteristics

Antibodies are glycoproteins organised from two light and two heavy chains, building a molecular Y-structure. The light chains have an N-terminal variable region and a C-terminal constant region. The complementary determining regions (CDR) defining the antibody specifity and the affinity towards its epitope are located within the variable sequences. The corresponding region on the antigen is the antigenic determinant or epitope. Intra-molecular disulfide bindings stabilize the different domains and an inter-molecular disulfide binding connects the light and the heavy chains with each other (Fig7).
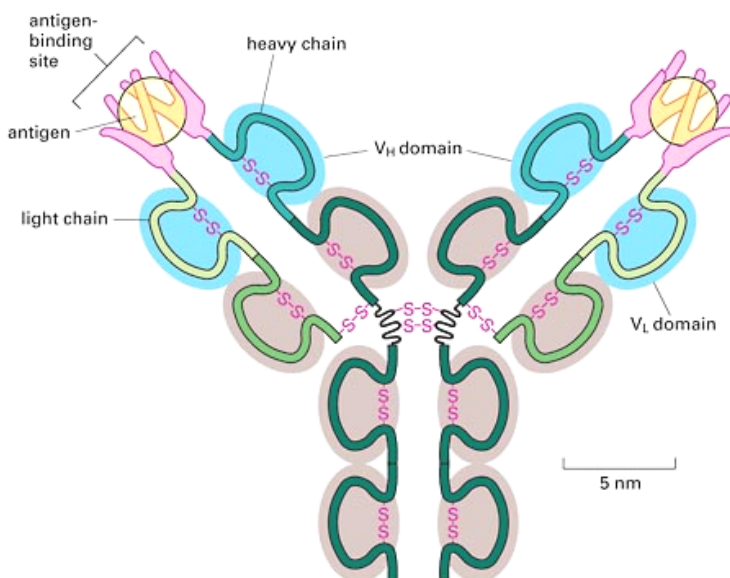
Figure 7 Schematic drawing of a typical antibody molecule. The protein is composed of four polypeptide chains (two identical heavy chains and two identical and smaller light chains) held together by disulfide bonds. Each chain is made up of several different domains, here shaded either blue or gray. The antigen-binding site (CDR) is formed where a heavy chain variable domain (VH) and a light chain variable domain (VL) come close together. Modified from Alberts et al., 1988

The binding of an antibody to its antigen is reversible and mediated by weak non-covalent forces, including hydrogen bonds, hydrophobic van der Waals forces, and ionic interactions. For an antibody-antigen interaction molecules have to be close enough allowing the antigen to interact with the complementary recesses on the surface of the antibody. Multivalent antigenic macromolecules are characterised by many different epitopes while polyvalent antigens consist of at least two or more identical epitopes, i.e. in a polymer with a repeating structure. The antigen (Ag)- antibody (Ab) interaction can be expressed as:

$$Ag+Ab \leftrightarrow AgAb$$
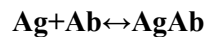
The equilibrium point depends both on the concentrations of Ab and Ag and on the strength of their interaction. Thus a larger fraction of Ab will become associated with Ag as the concentration of Ag increases. The strength of the interaction is generally expressed as the affinity constant (K_a):

$$K_a = [AgAb] \, / \, [Ag][Ab]$$

The affinity of an antibody for an antigenic determinant describes the strength of binding of a single copy of the antigenic determinant to a single antigen-binding site, and it is independent of the number of sites. The avidity describes the total binding strength of a polyvalent antibody with a polyvalent antigen.

Immunoglobulins can be subdivided into the classes of IgM, IgG, IgA, IgD and IgE depending on their molecular size and their immunological function:
IgM antibodies are secreted in the early phase of immunoglobulin production in infection and mediate the activation of the complement system. Membrane anchored IgM molecules are localized in the cytoplasm membrane of precursor B-cells functioning as antigen receptors. Secreted IgM antibodies are organised in a pentamer structure stabilized by a J-peptide connecting the Fc parts of the molecules. They show a relatively weak affinity towards its epitopes compensated by a high avidity based on its multimerised structure.

IgD antibodies are similar to IgM produced in the early phase of infection and have a membrane anchored counterpart. It is supposed that IgD antibodies are involved in the differentiation of precursor B-cells to plasma cells.

IgG antibodies represent the main population of antibodies in the serum. They have a key function in protective immunity after repeated exposition of an antigen. The IgG antibody class is the most specific one and can be subdivided into several IgG-subclasses. Due to distinct immunological functions, the induction of IgG subclasses is based on the type of contaminant being exposed in the organism. Viral infections predominantly lead to the production of IgG_1 and IgG_3, capable of activating the complement system. IgG_1 is mainly induced by the exposition of bacterial

polysaccharids. IgG specific Fc receptors can be found on macrophages, monocytes and neutrophile granulocytes binding the antibody-antigen complexes in order to phagocyte them. Further more the ADCC reaction is mediated by IgG antibodies.

IgA antibodies are produced in secretory organs and are predominantly involved into the humoral immune response in mucosal tissue. IgA antibodies are organised in a dimer structure stabilised by a J-peptide connecting the Fc parts of the molecules.

IgE antibodies are the major component of the humoral immune response against parasites. With the Fc receptor IgE antibodies can bind to basophile granulocytes and mast cells inducing the secretion of histamines. IgE molecules display a critical component of allergetic reactions and thus can mediate an allergetic shock reaction.

B-cells develop from pluripotent stem cells of the bone marrow and differentiate under the influence of cytokines to precursor B-cells. An ongoing differentiation towards pre-B-cells is induced by IL4, IL5 and IL6. During this process a somatic recombination occurs in the B-cells. This recombination leads to the reorganisation of genes encoding for V, D and J segments displaying the variable domains of the immunoglobulins (VDJ recombination). By that mechanism several million possibilities of VDJ combinations lead to a specifity for almost any antigen. In that phase, membrane anchored IgM molecules serve as antigen receptors and initiate the endocytosis of antigen-antibody complexes into B-cells. The phagocyted antigen is processed in the cellular proteasome and resulting peptides bound to MHC-II complexes are presented on the cell surface. Specific T-cells bind the MHC-II presented antigen by their T-cell receptor and initiate the differentiation of B-cells to antibody secreting plasma cells by releasing different cytokines. A change of the predominant immunoglobulin class occurs depending on cytokine signalling (switch). Alternatively processes of mRNA splicing rearrange the variable domains of the heavy chains and lead to the translation of antibody classes with higher affinities for the antigen, i.e. IgG classes. Within the V-segments encoding for the highly variable CDR domains hypermutations occur. These result in the production of highly specific antibodies with high affinities to their specific antigens. The selection process of affinity maturation of antibodies is mediated by repeated hypermutations as a result of ongoing antigen exposition. Thus antibodies evolve increased affinities for their antigens during the course of infection.

1.2.6 Neutralising antibodies

Neutralising antibodies are able to prevent the absorption of viral particles to their cellular receptor blocking the entry of the virus. It has been shown that in lentiviral replication cycles neutralising antibodies can decelerate the infection (Haynes 1992). It is supposed that neutralising antibodies limit

the replication of lentiviruses by the observation that escape mutants are generated resistant to neutralisation (Albert et al., 1990). In HIV-1 infected patients high titres of these antibodies appear to decelerate HIV-pathogenesis (Reitz et al., 1988, Nara et al., 1990, Watkins et al., 1996, Parren et al., 1999, Dianzani et al., 2002). Until today only four broadly neutralising monoclonal antibodies have been isolated from HIV-1 infected patients. IgG_1 2G12 and IgG_1 b12 bind to gp120, IgG_1 2F5 and IgG_1 4E10 bind to the ectodomain of gp41. Others have been described with a less effective capacity in broad neutralisation of HIV-1 subtypes (fab fragment X5, IgG447-52D and 17b against gp120 and Z13 against gp41).

1.2.7 The HIV-1 neutralising monoclonal antibodies 2F5 and 4E10

The mAb2F5 (Muster et al., 1993) and the mAb4E10 (Zwick et al., 2001a) are produced in hybridoma cell lines generated by fusion of B-lymphocytes of an HIV-1 positive donor with a human tumor cell line (Buchacher et al., 1994). The encoding gene for 2F5 shows an abnormality within its D-segment for the heavy immunoglobulin chain by a length of 52 nucleotides (Kunert et al. 1998). This unusual length is supposed to result from recombination processes and leads to a relatively long CDR3 loop of the 2F5 immunoglobulin. 2F5 is capable of neutralising laboratory as well as primary isolates of group M and shows an in *vitro* neutralisation at a $TCID_{50}$ of <1µg/ml in dependence of the subtype being neutralised (Conley et al., 1994, Muster et al., 1994, Purtscher et al., 1994, Trkola et al., 1995, Purtscher et al., 1996). 2F5 binds to the linear sequence ELDKWA (AS 662-667, HIV- reference genome HXB2, NCBI K03455, Ratner et al., 1985). The epitope is located within the C-terminal region of gp41, displaying a domain being highly conserved in subtypes of group M (Zwick et al., 2001) and thus explaining the broad spectrum of neutralisation by 2F5. The core of the epitope (L)DKWA is supposed to be essential for neutralisation (Muster et al., 1993, McGaughy et al., 2003) due to the fact that amino acid deletions therein, i.e. isolates of group O, result in a loss of function (Trkola et al., 1995, Parren et al., 1998).

The 4E10 epitope NWFN/DIT is located three amino acids downstream of the 2F5 epitope and was original produced as an IgG_3 antibody. An exchange of the subclass specific constant immunoglobulin regions to an IgG_1 molecule (Kunert et al., 2000) resulted in an increased capacity of neutralisation and subtype specifity (Stiegler et al., 2001). Similar to 2F5 also 4E10 neutralises HIV-1 laboratory and primary subtypes of group M.

The combination of 2F5 and 4E10 with other neutralising antibodies showed a synergistic effect increasing neutralising efficacy (Li et al., 1997, Mascola et al., 1997, Zwick et al., 2001, Kitabwalla et al., 2003). The injection of high dosis of different neutralising antibodies into HIV-1 infected patients showed a decline in virus burden (Stiegler et al., 2002, Armbruster et al.2002). Despite numerous studies focussing on the induction of 2F5-like antibodies the induction of broadly neutralising antibodies towards the gp41 C-terminal domain was not successful until today. In addition to the

ELDKWA and the NWFN/DIT epitopes it was recently published that 2F5 as well as 4E10 should bind to cardiolipin suggesting a polyspecific autoreactivity for these antibodies (Haynes et al., 2005). Thus the question arose if an autoantigen mimicry of the conserved membrane-proximal epitopes of the virus prevents the induction of 2F5- and 4E10-like antibodies.

1.2.8 Neutralising antibodies in the course of FeLV infection in cats

In case of FeLV virus neutralising antibodies appear to correlate with immunity to FeLV (Russel & Jarrett 1978, Hoover et al., 1978). They are considered being mediated mainly by the induction through the FeLV surface glycoprotein gp70. Cats that resist infection also develop antibodies to the so-called feline oncornavirus-associated cell membrane antigen (FOCMA). Anti-FOCMA antibodies have been associated with protection from FeLV related neoplastic disease, but their role in protection from persistent viraemia is less clear (Rojko & Hardy 1994). Of 21 cats that did not develop virus neutralising antibodies after challenge in one study, 20 (95%) became persistently viraemic compared to only three of the 24 (13%) that did develop virus neutralising antibodies (Grant et al., 1980). The presence of neutralising antibodies was shown to be clearly associated with resistance to infection since passive transfer of antibodies either naturally through the colostrum (Hoover et al., 1977, Jarrett et al., 1977), or experimentally by infusion (Haley et al., 1985) protects cats against FeLV challenge.

1.3 Vaccine development against retroviruses

1.3.1 Vaccination strategies

At least four different main vaccination strategies exist based on the immunisation with (i) attenuated viruses, (ii) inactivated viruses, (iii) virus derived protein and peptide constructs and (vi) DNA constructs encoding viral genes. (i) Attenuated viruses differ from the wild type virus by a decreased replication efficacy and pathogenicity. They are able to activate the humoral as well as the cellular immune response and lead to the induction of neutralising antibodies. The success of attenuated virus vaccines against diseases such as smallpox, measles and mumps has dramatically reduced morbidity and mortality worldwide and has led to the eradication of smallpox (Arita, 1979). However the use of an attenuated virus vaccine against SIVmac in neonatal macaques, deleted for the *nef* gene (SIVmacΔ*nef*), induced a persistent infection resulting in AIDS symptoms (Baba et al., 1995, Cohen et al., 1997). This data questioned the safety of an attenuated HIV-1 vaccine. Further more an attenuated HIV-1 vaccine also bears the possibility of gain of function mutations or insertion mutations by an integrated retroviral genome in vaccinated patients. (ii) Inactivated virus particles are used in vaccines against influenza and polyomeyelitis. They are based on wild type viruses inactivated by chemicals

maintaining conformational structures of viral proteins but degenerating viral nucleotides. (iii) Usage of virus derived protein or peptide components in vaccines eliminate a possible contamination with infectious particles. In general viral surface proteins display critical targets for vaccination. In case of the hepatitis B-virus its recombinant surface protein HbsAg is used inducing a neutralising antibody and a CTL response in vaccinated humans. (iv) DNA vaccines primarily initiate a CTL response (Lemieux, 2002). Applicated DNA is absorbed by cells and encoded proteins are expressed and presented by MHC-I molecules on the cell surface.

1.3.2 Vaccination with recombinant proteins can provide protection from retroviral infections

Immunisation is the most effective method to prevent diseases caused by infectious agents. It is of interest that most, if not all, successful vaccines are based on the induction of neutralising antibodies. Although the development of vaccines against HIV, the retrovirus that causes AIDS, is one of the major task of present vaccinology, all attempts have until now failed. Approximately 40 million individuals worldwide were living with HIV in 2004 and more than 28 million have died since the pandemic began. Although there are therapies available that prevent or delay the onset of AIDS, there is at present no cure for the infection. Present therapies are based mainly on combinations of inhibitors of the viral reverse transcriptase and of the protease. In addition, fusion inhibitors such as T20, that corresponds to a conserved domain of the transmembrane envelope protein gp41 of HIV-1 and can prevent infection, have been included in combination therapies (Turpin, 2003).

In contrast, vaccines against feline leukaemia virus (FeLV) not only exist but are also commercially available, showing that antiretroviral vaccines are not impossible. The number of cases of FeLV-induced disease has been reduced by the development and use of several vaccines against FeLV-A. However, none of the seven commercial FeLV vaccines currently available in the USA and Europe provide 100% protection against infection. Three vaccines are composed of inactivated whole virus, two are gp70 subunit vaccines and two are recombinant vaccines (Sparkes, 1997). The Leucogen (Virbac) vaccine contains the recombinant unglycosylated p45 of the surface envelope glycoprotein gp70 and is one of the most effective (Jarrett & Ganiere, 1996).

Table 2 Marketed vaccines against FeLV-A

Vaccine	Company	Multiple Vaccine	FeLV component	Laboratory Animals in Studies	PF* (%)	Reference
Eurifel FeLV	Merial	-	canarypox virus expressing *env, gag of FeLV-A*	20	93.2%	(Hoover et al., 1996)
Eurifel RCP / FeLV	Merial	+	canarypox virus expressing *env, gag of FeLV-A*	-	-	-
Fevaxyn FeLV	Fort Dodge	-	subunit filtrate containing inactivated feline leukaemia virus (strain 61 E)	144	90.4%	(Hines et al., 1991)
Fel-O-Vax	Fort Dodge	-	inactivated feline leukaemia virus (strain FeLV-61E-A)	12	86%	(Jarrett & Ganiere 1996)
Purevax Leucat	Merial	-	inactivated feline leukaemia virus	12	14.3%	(Jarrett & Ganiere 1996)
Leucocine	SmithKline	-	mixed subunit filtrate containing feline leukaemia virus antigen gp 70 (1330ng)	-	-	-
Leukocell 2	Pfizer	-	mixed subunit filtrate containing feline leukaemia virus antigen gp 70 (1330ng)	148	74,8%	(Pollock & Haffer 1991)
Leucogen	Virbac	-	purified recombinant p45	12	52,4%	(Jarrett & Ganiere 1996)

*preventable fraction

1.3.3 FeLV is a useful model to explore the factors that should be taken into account in developing a successful HIV vaccine

Feline leukaemia virus (FeLV) is a gammaretrovirus comprising three subtypes A, B and C (Jarrett 1975) of which FeLV-A is of veterinary importance, being the predominant serotype in cats (Jarrett & Hardy 1978). FeLV infection was, until recently, the most common fatal disease of cats.

Cats exposed to FeLV may either become persistently viremic or recover from infection. Viremic cats show little evidence of an immune response to the virus and are at high risk of developing a fatal disease within 2 to 4 years. In contrast, recovered animals produce virus neutralising antibodies and FeLV-specific cytotoxic T-cells (CTLs), are resistant to reinfection, and do not develop FeLV-related diseases (Hardy et al., 1980). A third possible outcome is the establishment of a latent infection, in which cats are not viremic but have a covert infection of bone marrow cells and, like fully recovered cats, have virus-neutralising antibodies (Madewell & Jarrett 1983). In most cats with latent infection the virus is eventually eliminated, but occasionally the infection persists for several years (Pacitti & Jarrett 1985) and may be reactivated at a later date so that cats become viremic. Co-infection with the immunosuppressive feline immunodeficiency virus (FIV) has been shown to reactivate FeLV (Hofmann-Lehmann et al., 1997).

FeLV causes different fatal diseases, among them leukaemia. Leukaemia was the first disease associated with FeLV and, thus, the source of its name. However, more cats die from immunosuppression induced by FeLV than from leukaemia and these immunodeficiencies resemble in

many parameters AIDS induced by HIV in humans or by FIV in cats. This includes a decrease in the number of CD4+ cells (Hofmann-Lehmann et al., 1997) and numerous opportunistic infections (Hardy 1993, Hardy et al., 1976). Although all three viruses are retroviruses, they belong to a different genus (FeLV is a gammaretrovirus, HIV and FIV are lentiviruses) and differ in their morphology and genetic composition. Nevertheless, the experience of successful vaccination against FeLV is of great interest when developing strategies for an AIDS vaccine. FeLV and FIV (which is more closely related to HIV) provide useful systems to explore the factors that should be taken into account in developing successful retroviral vaccines for an outbred population.

1.4 Goals of this study-Targeting at FeLV-A and HIV-1

The transmembrane envelope proteins of retroviruses are very similar in their structure due to their specific functions during infection. Conformational changes include an intramolecular interaction between a N-terminal and a C-terminal helix region (Follis et al., 2002). Although the surface envelope proteins gp70 of gammaretroviruses are not as variable as the surface envelope protein gp120 of HIV-1, the transmembrane envelope proteins of all retroviruses are more highly conserved and therefore possibly represent a better antigen for vaccination.

The aim of this study was to analyse the humoral immune response against the ectodomain of FeLV-A p15E in different species in order to establish a model for the immunological reaction against retroviral transmembrane proteins. Eventually induced neutralising antibodies should be determined in order to evaluate the effectiveness of a transmembrane protein based vaccine against retroviruses. Further more a potential vaccine candidate should be analysed in comparison to commercial vaccines against FeLV. The FeLV-A model also allows to test the effectiveness of a possible vaccine candidate in an *in vivo* analysis by immunisation and virus challenge of cats. In comparison to previously published data (Fiebig et al., 2003) describing the immune response against the p15E of the porcine endogenous retrovirus firstly possible similarities in the humoral immune reactions within the genus of gammaretroviruses should be determined. Based on the data obtained from these immunisation attempts a concept should be worked out for the construction of a recombinant gp41-derived protein inducing neutralising antibodies against HIV-1. This concept should consider main aspects found for the model of the immune response against gammaretroviruses.

2. Materials and Methods

2.1 Chemicals

If not indicated otherwise all chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany)

2.2 Cloning and expression of recombinant proteins used for immunisations

2.2.1 FeLV-A p15E ectodomain

DNA from FeLV-A producing FEA feline embryonic fibroblast cells was isolated using a Qiagen DNA isolation kit. Using the forward primer FeLV-A p15E forw. and the reverse primer FeLV-A p15E rev., a sequence corresponding to the ectodomain of the transmembrane envelope protein p15E (amino acids 476-583) was amplified by polymerase chain reaction and cloned into the pCal-n vector (Stratagene, Europe, Amsterdam, Netherlands). *E. coli* BL21 DE3 cells were transformed and p15E N-terminally fused to a 4 kDa calmodulin binding protein (CBP) was produced. The fusion protein was purified by calmodulin resin affinity chromatography (Stratagene). Protein to be used for immunisation and for inhibition experiments was extensively dialysed against phosphate-buffered saline (PBS).

2.2.2 ΔISU p15E and p15E/gp41 I and II hybrid proteins

The p15E backbone was amplified from the pCaln-p15E vector (aa476-512 and aa530-539 and 558-583). The DNA sequence of the immunosuppressive domain (Cianciolo et al., 1985; Denner 1998) and the peptide J and K (Nick et al., 1990) were deleted by multi step PCR. Using primers FeLV-A forward and primer p4 fragment 1 corresponding to aa476-512 and 530-539 was amplified containing an N-terminal *Bam* HI restriction site and a C-terminal *Not* I restriction site. The fragment 1 obtained from that PCR is deleted for the DNA sequence of isu (aa513-529) and bears the DNA sequence of the cystein loop (aa531-539). Using primers p5 and FeLV-A reverse fragment 2 corresponding to aa558-583 was amplified lacking the C-terminal opposing sequence of isu (aa540-558). The fragment 2 contained an N-terminal *Not* I restriction site and a C-terminal *Eco* RI restriction site.

The p15E/gp41 I and II hybrid proteins consist of fragment 1 obtained for the generation of FeLV-A ΔISU p15E (2.2.1) and the DNA sequence of the E2 region of HIV-1 IIIB gp41 amplified from the pNL4-3 vector (aa645-aa680 for p15E/gp41 I and aa636-aa680 for p15E/gp41 II). For the amplification of the gp41 E2 sequences the gp41 reverse primer and p5 hybrid I or p5 hybrid II primers were used generating an N-terminal *Not* I restriction site and a C-terminal *Eco* RI restriction site.

The amplified sequences for FeLV-A ΔISU p15E and for the p15E/gp41 hybrid proteins I and II were ligated using inserted restriction sites *Not*I, *Bam*HI and *Eco* RI and cloned into the pCal-n vector

(Stratagene, Europe, Amsterdam, Netherlands). *E. coli* BL21DE3 cells were transformed and the hybrid protein fused to the 4 kDa calmodulin binding protein (CBP) was produced.

Due to the insolubility of recombinant proteins FeLV-A ΔISU p15E, p15E/gp41 hybrid protein I and II calmodulin resin affinity chromatography was inefficient. Thus the resulting *E.coli* pellet, containing the hydrophobic target protein, was washed intensively, air dried, pulverised and suspended in PBS.

2.3 Experimental animals

2.3.1 Immunisation of rats and goat with the FeLV-A p15E ectodomain

Wistar rats were obtained from a commercial supplier and housed in groups under barrier conditions. To generate p15E-specific antibodies, 12 rats and one goat were immunised intramuscularly (i.m.) and subcutaneously (s.c.) three times (at 0, 3 and 6 weeks unless stated otherwise) with 0.5 mg of the affinity-purified recombinant fusion protein in 0.4 ml buffered saline emulsified in 0.4 ml incomplete Freund's adjuvant.

2.3.2 Immunisation of rats with ΔISU p15E and p15E/gp41 I and II hybrid proteins

Wistar rats were obtained from a commercial supplier and housed in groups under barrier conditions. Rats were immunised intramuscularly (i.m.) twice (at 0 and 3 weeks unless stated otherwise) with 0.1 mg recombinant protein in 0.5ml buffered saline emulsified in 1.2 ml Montanide® ISA 720 (Seppic, France, lot number 143521). Three rats were immunised with FeLV-A ΔISU p15E (group70), 10 rats were immunised with p15E/gp41 hybrid protein I (groups 71, 79 and 91) and 4 rats were immunised with hybrid protein II (group 90). Additionally 3 rats were immunised with p15E/gp41 I hybrid protein as described and boostered once with 0.1mg gp41-derived peptide (aa656-680) emulsified in 0.25ml Montanide® ISA 720.

2.3.3 Immunisation of rats with p15E and p45

Two immunisation experiments were performed using Wistar rats. In the first experiment, 9 rats were immunised twice intramuscularly (i.m.) and subcutaneously (s.c.) (at weeks 0 and 3). For immunisation recombinant p15E of FeLV-A was prepared as described. In the first experiment 1 dose Leucogen, containing 0.1mg p45 plus Quil-A and aluminium hydroxide as adjuvant (Virbac, lot number 80986902143521) was given alone or mixed with 0.5mg p15E. Freund´s adjuvant was used when p15E was injected alone. In the second experiment 18 rats were immunised i.m. and s.c. with 0.1mg or 0.5mg p15E alone, with one dose Leucogen p45 alone or with a mixed preparation of 0.1mg

or 0.5mg p15E and one dose Leucogen p45 at week 0 and 3. Immunisation with p15E alone was performed at a dilution 7:3 in Montanide® ISA 720 (Seppic, France, lot number 143521).

2.3.4 FeLV-A infected pet cats, immunisation of cats with p15E and p45

Sera from infected cats were obtained from household cats in Germany at the time of first diagnosis of infection using a commercial p27 Gag antigen detection assay (Feline leukemia virus antigen test kit, Symbiotics, USA).

6-10 month old cats, obtained from the University of Düsseldorf and housed in groups of 3, were immunised intramuscularly (i.m.) twice (at weeks 0 and 3). For p15E immunisations Montanide® ISA 720 (Seppic, France, lot number 143521) was used as adjuvant mixed with p15E at a ratio of 3:7. 6 cats were immunised with 0.5mg p15E, 3 cats were immunised with one dose Leucogen® p45 each (Virbac, lot number 80986902143521) and 2 cats were immunised with a mixed preparation of 0.5mg p15E and one dose Leucogen® p45. A group of 3 cats did not receive any immunisation serving as positive control group. The cats were challenged oronasal with 4 doses of 1×10^6 ffu/ml FeLV-A Glasgow strain (kindly provided by Dr, O. Jarrett, Department of Veterinary Pathology, University of Glasgow Glasgow, UK) at four consecutively days. Blood was taken 3 days before challenge and 10, 30, 60, 80 and 100 days after challenge.

2.4 Purification of antisera by p15E affinity and proteinG columns

Purified p15E coupled to CNBr-activated Sepharose (Pharmacia) was used to adsorb specific antibodies from serum that were then eluted using Tris-glycine buffer, pH 2.5 and dialysed against PBS. Protein G columns (Montage®, Millipore) were used in parallel to purify IgG as described by the manufacturer.

2.5 Characterisation of antisera in Western blot and ELISA

SDS-PAGE and Western blotting were performed as described previously (Tacke et al., 2001) using 1µg-20µg of purified recombinant protein per lane. Pre- and post-immunisation experimental animal sera were titrated in ELISA using as antigen affinity-purified recombinant p15E protein, FeLV-A ΔISU p15E protein, Leucogen p45, p15E/gp41 I hybrid protein or HIV-1 gp41 E2 peptide (aa656-680). Plates (Nunc) were coated for 1h at 37°C or in case of Leucogen® p45, p15E/gp41 I hybrid protein and HIV-1 gp41 E2 peptide at 37°C for 18 hours with protein diluted in PBS (100ng/well). ELISA plates then were washed once with PBS containing 0.1% Tween 20 and blocked for 1 h at room temperature with PBS containing 0.1% Tween 20 and 5% BSA. Serum samples, diluted in PBS containing 2.5% BSA and 0.1% Tween-20, were added to the ELISA plate at an initial dilution of

1:100 or 1:1000 and diluted further in three- or four-fold steps. After incubation for 1h at 37°C, ELISA plates were washed three times in PBS containing 0.1% Tween 20 and a horseradish peroxidase conjugated secondary antibody specific for human IgG (Sigma-Aldrich), goat IgG (Dako), rat IgG (Dako) or cat IgG (Bethyl, USA), diluted 1:2000-1:8000 in PBS containing 2.5% BSA and 0.1% Tween-20, was added. Incubation for 1h at 37°C was followed by five washes with PBS containing 0.1% Tween 20. Finally, ELISA plates were developed by addition of OPD (a-phenylenediamine dihydrochloride) diluted in PBS (50µg/well) plus 0.1 % H_2O_2 and stopped after 10 minutes by addition of 30µl H_2SO_4 (5N). Antibody endpoint titres are reported as the dilution giving an $OD_{492/620nm}$ reading above the background of pre-immune sera. Protein-specific antibody endpoint titres are reported as the dilution giving an $O.D._{492/620nm}$ reading above that of preimmune sera.

2.6 Epitope mapping on FeLV-A p15E and HIV-1 gp41

Peptides corresponding to the entire p15E of FeLV-A, Glasgow strain or to the entire gp41 of HIV-1IIIB were synthesized as 15-mer peptides overlapping by 13 amino acids and were covalently linked to a cellulose sheet (Jerini Biotools). Sera diluted 1:500-1:1000 were incubated with the membrane for 2h, washed three times for 15 min with Tris-buffered saline, pH 7.5 containing 0.05% Tween 20 (Sigma) and incubated for 2 h with a peroxidase-conjugated secondary antibody diluted 1:3500-1:10,000. Binding was detected using a chemiluminescence detection solution (ECL, Amersham Pharmacia Biotech).

2.7 Immunofluorescence on FEA cells

FeLV-A producing FEA cells were grown on chamber slides, washed three times with PBS and fixed with 3.5% formaldehyde. Unspecific binding sites were blocked with 5% BSA in PBS for 20 minutes followed by washing with PBS. Cat sera were applied in 2.5% BSA/PBS at a dilution of 1:1000 and incubated at 37°C for 1 h. After five washes with PBS the cells were incubated with FITC-labeled goat anti-cat IgG (Bethyl, USA). Finally, the cells were embedded in Prolong® antifade reagent (Molecular Probes) and the surface fluorescence was analysed by confocal microscopy (Zeiss, LSM510). Unspecific cell fluorescence at 543nm was subtracted from the specific signal at 488nm.

2.8 Retroviral neutralisation assays

2.8.1 FeLV-A neutralisation assay with FEA cells

The virus stock for the neutralisation assay was prepared as cell-free supernatant from FEA cells infected with the FeLV-A Glasgow strain (kindly provided by M. Reinacher, Giessen, Germany and O. Jarrett, Glasgow, UK). The stock was titrated on uninfected FEA cells and shown to have a titre of $10^{4.76}$ $TCID_{50}$/ml. Neutralisation assays were performed as follows. FEA cells were seeded at 6000 cells per well into 96-well microtitre plates one day before use. Preimmune and immune sera were heat-inactivated at 56°C for 30 min. 50μl of virus was added to serial dilutions of serum or purified immunoglobulin and incubated for 30 min at 37°C before transfer to the cells. Alternatively, the dilution of serum was held constant at 1:5 and the virus was serially diluted. After 3 days incubation, cells were freeze-thawed three times and a lysis buffer containing 20 mg/ml of proteinase K in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 8.4) was added. The cells were incubated for 3h at 56°C followed by 10 min at 95°C to inhibit proteinase K activity. Provirus was then quantitated by PCR as described in 2.9.1.

2.8.2 HIV-1 and PERV virus neutralisation assays with C8166 cells

The virus stock for the neutralisation assay was prepared as cell-free supernatant from C8166 cells infected with HIV-1IIIB and titrated on uninfected C8166 cells. 50μl cell-free virus-containing supernatant ($1x10^3$ $TCID_{50}$) were used to infect $5x10^4$ C8166 cells seeded into a well of a 96 U-well microtitre plate in a total volume of 100μl. Preimmune and immune sera were heat-inactivated at 56°C for 30 min. Serial dilutions of the sera were added to the virus and incubated for 45 min at 37°C before transfer to the cells. After 72 hours incubation (37°C, 5% CO_2), cells were freeze-thawed three times and a lysis buffer containing 20 mg/ml of proteinase K in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 8.4) was added. The cells were incubated for 3h at 56°C followed by 10 min at 95°C to inhibit proteinase K activity. Provirus integration in infected cells was measured by quantitative real-time PCR as described in 2.9.2. Neutralisation assays on macrophages with HIV-1 isolates Bal and SF162 were carried out on differentiated macrophages as described previously (Ruppach et al., 2000) and subsequently analysed as described above. For neutralisation assays performed with immunoglobulins, protein G columns (Montage®, Millipore) were used to purify IgG as described by the manufacturer.

For infection of C8166 cells with PERV 50μl cell-free virus-containing supernatant of PERV/5°-infected 293 cells ($1 \times 10^{4.31}$ $TCID_{50}$/ml) was added to the cells and identical procedures were carried out as described for HIV-1 neutralisation assay. Quantitative real-time PCR analysis for PERV provirus integration was performed as described in 2.9.3.

2.9 Determination of retroviral provirus integration by real time PCR

2.9.1 FeLV-A real time PCR

An internal probe FAM-5´-TTAAGCACCTGGGCCCCGGC-3´-DQ (Eurogentec) was used together with FeLV-specific primers. The sense primer 5´-TCAAGTATGTTCCCATGAGATACAA-3´ and antisense primer 5´-GAAGGTCGAACTCTGGTCAACT-3´ were used to amplify and to quantify a 185bp product from the exogenous U3 sequence in the LTR region of the FeLV-A provirus genome. The 25µl reaction mixture consisted of 1x PCR buffer with 1mM $MgCl_2$, 0.5µM each of dATP, dCTP, dGTP, dTTP, 5pmol of each primer, 5pmol of probe, 1.25 U Amplitaq Gold polymerase and 2µl lysis mixture obtained from the FeLV-A neutralisation assay (2.8.1). The thermal cycling conditions used were 12 minutes at 95°C followed by 50 cycles of 1 minute at 95°C, 1 minute at 59°C and 30 seconds at 72°C in a Stratagene MX4000 machine if not indicated otherwise.

2.9.2 HIV-1 real-time PCR

2µl of cell lysate from neutralisation assays were used as template in a gp41-specific real-time PCR using primers SK68i (5´-GGARCAGCIGGAAGCACIATGG-3´), SK69i (5´-CCCCAGACIGTGAGI TICAACA-3´) and the probe 6Fam-TGACGCTGACGGTACAGGCCAGAC-dabcyl (Taqman Universal Mastermix, Applied Biosystems). The assay was performed using a Stratagene MX4000 (55cycles, annealing at 55°C, no elongation phase) (Schweiger et al., 1997).

2.9.3 PERV real-time PCR

2µl of cell lysate from neutralisation assays were used as template in a PERV gag specific real-time PCR using primers PERV real s (5'-TCCAGGGCTCATAATTTGTC-3´), PERV real_as (5'-TGATGGCCATCCAACATCGA -3´) and the probe 6Fam- AGAAGGGACCTTGGCAGACTTTCT -dabcyl (Taqman Universal Mastermix, Applied Biosystems). The assay was performed as described for HIV-1 real-time PCR (2.9.2)

2.9.4 Calculation of provirus integration and neutralisation efficiency

In order to obtain the percentage of provirus integration, the Δct value of the control serum and the immune serum was calculated and used in the formula $(2^{-\Delta ct})$ x100%. The FeLV-A, PERV and HIV-1 neutralisation efficacy was calculated as percentage of provirus integration subtracted from 100%.

2.10 Quantification of p27 antigen and provirus load in FeLV-A infected cats

Sera obtained from household cats or from FeLV-A challenged cats were tested for productive infection using a commercial p27 Gag antigen detection assay (Feline leukemia virus antigen test kit, Symbiotics, USA). Provirus load was determined by DNA purification from 100µl peripheral blood, analysed for provirus integration in a FeLV-A real time PCR and calculated in copies/µl blood by the usage of a FeLV-A plasmid standard.

2.11 Generation of hybridoma cells from rat lymphocytes

Shortly after the spleen was excised from a rat producing the antibody of choice, it was teased in ice cold serum-free medium (D-MEM without supplements) and the resulting cell suspension was passed through a Falcon 70 micron cell filter and suspended in 50 ml of ice cold D-MEM without supplements. The cells were centrifuged and washed three times at 4° C. Afterwards cells were resuspended in 10 ml D-MEM without supplements and viable cells were counted. The cells were kept on ice. Concurrently with the spleenocytes, the mouse myeloma cells P3X63Ag8.653 (maintained at $<1x10^6$cells/ml) were centrifuged and washed three times. They were resuspended in D-MEM without supplements and viable cells were counted. An appropriate number of myeloma cells were added to the entire volume of spleen cells in a ratio of 1:5 and centrifuged together. All supernatant was aspirated and the resulting pellet was suspended by tapping the end of the tube. The tube was placed in a container of warm water (37°C) and 1 ml of 37° C 50% w/v PEG was gradually added over a period of 60 seconds, while tapping the side of the tube to achieve thorough mixing. Additional 60 seconds the mixing was continued subsequently followed by diluting the PEG/cell mixture slowly by adding dropwise 2 ml of D-MEM without supplements over a 2 minute time span. Adjacent 8 ml 37° C D-MEM supplemented with 10% FCS, HAT, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 U penicillin, 50 µg streptomycin and 50 µM 2-ME were added over a 4 minute period and finally the total volume was equilibrated to 50 ml using D-MEM with supplements (20%FCS). The cells were centrifuged at 4°C and resuspended in medium at the appropriate volume to bring the cells to a concentration of $1.5x10^6$cells/ml. From this suspension 150 µl were added to each well of 6x96 well plates (Köhler and Milstein 1975).The day of the fusion was considered day 0 and the fusion plates were examined at 24-48 hours for any abnormalities (i.e. bacterial contamination). On day 7, wells were inspected visually and then fed with 150 µl of D-MEM with supplements (20%FCS) on days 7, 11 and thereafter as needed. The cultures were examined visually at each feeding. Once a majority of wells appear 50% confluent for growth, supernatants were harvested for screening in ELISA and cells were fed at this time. 21 days after fusion, the HAT component in the medium was replaced by HT and cells were transferred to 24well plates. Corresponding cells to hybridoma cell culture supernatants tested positive in screening ELISA were singularized twice to obtain monoclonal antibodies. Finally HT selection medium was replaced by D-MEM with supplements containing 10% FCS and positive hybridoma cells were maintained in T75 flask.

3. Results

3.1 Induction of neutralising antibodies against FeLV-A p15E in rats and goat

In order to evaluate the capacity of the FeLV-A transmembrane envelope protein p15E to induce neutralising antibodies ten rats and one goat were immunised with p15E and the humoral immune response was analysed for specifically induced antibodies.

3.1.1 Characterisation of the antigen

In order to produce recombinant FeLV p15E for immunisation, DNA corresponding to the ectodomain of p15E (amino acids 476-583) (Fig.8A) derived from FeLV-A-producing FEA (feline embryonic fibroblast) cells was PCR amplified, cloned and the sequence verified. The protein was expressed in *E. coli* BL21 DE3 cells and the fusion protein containing p15E N-terminally fused to a 4kDa calmodulin binding protein (CBP) was purified by calmodulin resin affinity chromatography (Stratagene) and characterised by SDS-page. The protein was shown to have a molecular mass of 15 kDa compared to the 18 kDa of the viral p15E (Fig. 8B) and is two amino acids longer than the recombinant p15E of PERV, which was also produced as a CBP-fusion protein. It is important to note that both the recombinant and the viral FeLV p15E are not only recognised by goat serum induced by immunisation with the recombinant FeLV p15E, but also by serum specific for the recombinant PERV p15E (Fig.8B).
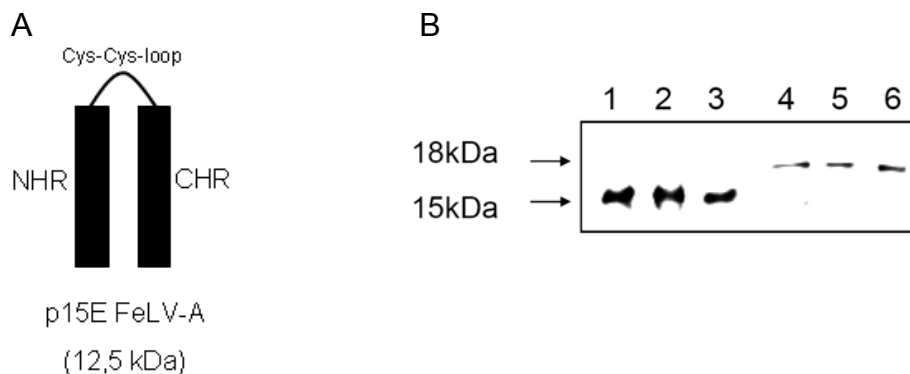
Figure 8 (A) Schematic model of the FeLV-A p15E ectodomain without CBP. Indicated are the cystein loop (Cys-Cys-loop) and the N- and C-terminal helix regions (NHR, CHR). **(B)** Western blot analysis of goat antiserum specific for FeLV-A p15E (1, 4), of affinity purified antibodies (2, 5) and of a goat antiserum specific for PERV p15E (3, 6). Antigens used were 1µg of recombinant FeLV-A p15E (lane 1-3) and 100µg of a cell lysate from FeLV-A producing FEA cells (lane 4-6). The Western blot was performed in a multiscreen blot chamber that allows the testing of multiple antisera in parallel.

3.1.2 Characterisation of binding antibodies

Immunisation of 12 rats and one goat with the FeLV p15E resulted in the generation of antisera that recognised the protein in Western blot analysis (Fig. 9). These sera also reacted with the recombinant protein in ELISA and with viral p15E in Western blot (Fig. 8B). Antibody titres ranged from 2.5×10^5 to $\geq 1 \times 10^6$ (Fig. 10, Tab.3). The preimmune sera did not react in any assay.
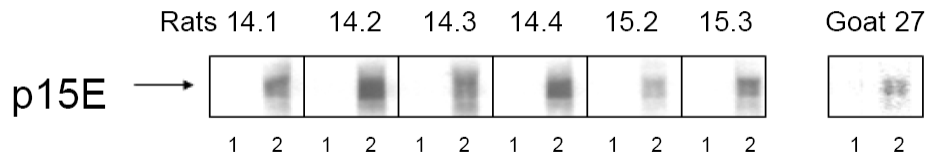
Figure 9 Western blot analysis of rat and goat antisera following immunisation with FeLV-A p15E. The antigen used was the same recombinant p15E ectodomain used for immunisation. The number of the serum tested is indicated at the top. Lane 1 shows the preimmune serum, lane 2 the corresponding immune serum.
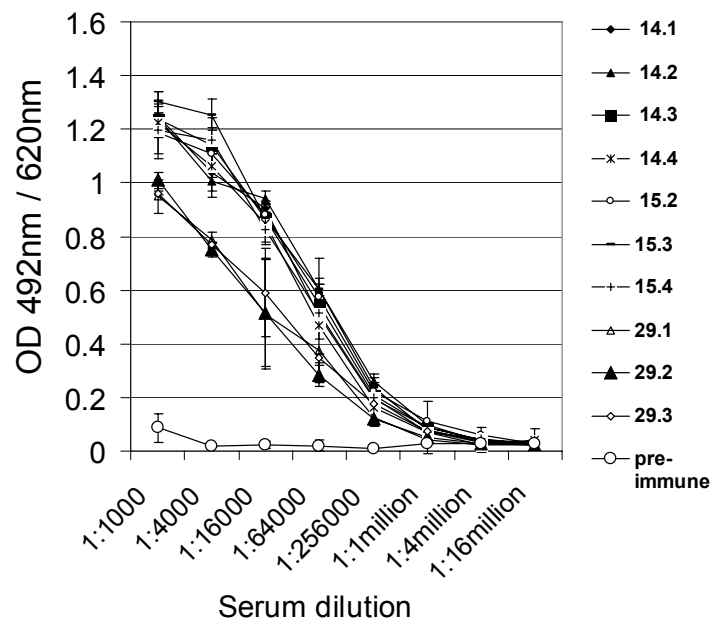
Figure 10 Determination of the ELISA titres of rat and goat antisera using the recombinant ectodomain of p15E as antigen. Immune sera and preimmune sera are indicated on the right.

3.1.3 Neutralising antibodies against p15E

All sera were able to inhibit the infection of feline embryonic fibroblast cells by the FeLV-A Glasgow strain, whereas the preimmune serum had no such neutralising activity. At a 1:5 dilution the rat sera were able to inhibit infection by 50% - 98% and the goat serum up to 99% (Tab.3). Antibodies purified from these sera by p15E or protein G affinity chromatography also neutralised in a dose-dependent fashion (Fig. 11) indicating that the activity was indeed based on antibodies and not on complement or other soluble antiviral factors.
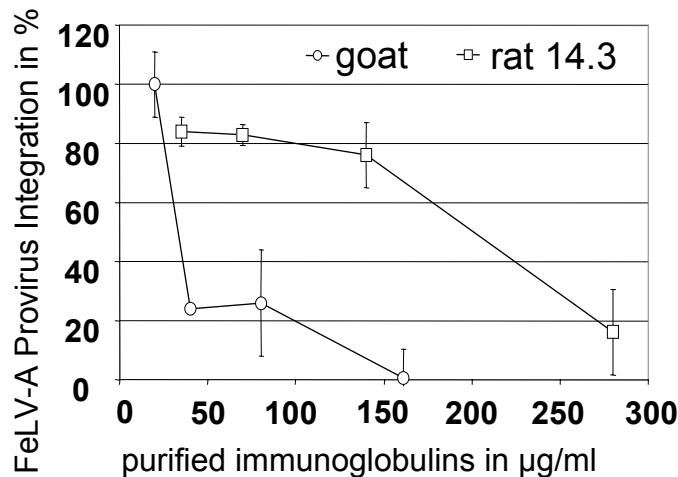
Figure 11 Neutralisation of FeLV-A Glasgow strain by p15E affinity purified immunoglobulins from goat serum 27 and protein G purified IgG from rat serum 14.3. Infection was measured by real time PCR detecting provirus integration and indicated as percentage of the signal obtained with infected cells treated with preimmune sera.

3.1.4 Epitope mapping

Linear 15-mer peptides (overlapping by 13 amino acids) corresponding to the entire FeLV-A p15E and bound covalently by the C-terminus to a cellulose membrane were tested for recognition by the various sera. With rat serum 15.3 three epitopes were detected (Fig. 12): KALLETAQF, located at the N-terminal end, ALEESISALEK, located near the first epitope and WFEGWN located N-terminal of p15E. Testing the sera of all 10 rats (rats 15.1. and 29.4 had died for unrelated reasons), confirmed these results and identified a fourth group of epitopes located in the region MAKLRERLKQRQQL. To summarise, four main epitope regions were found, two at the N terminus of the ectodomain designated E1a (LETAQFRQL) and E1b (ALEESISALEK) and two others at the C terminus of the ectodomain, designated E2a (MAKLRERLKQRQQL) and E2b (FDSQQGWFEGWFN) (Fig. 13, Tab.3). Although serum from rat 14.3 recognised only E1b and E2b, the purified IgG was able to recognise an additional epitope (E2a) suggesting accumulation and concentration of certain IgG's during affinity chromatography.
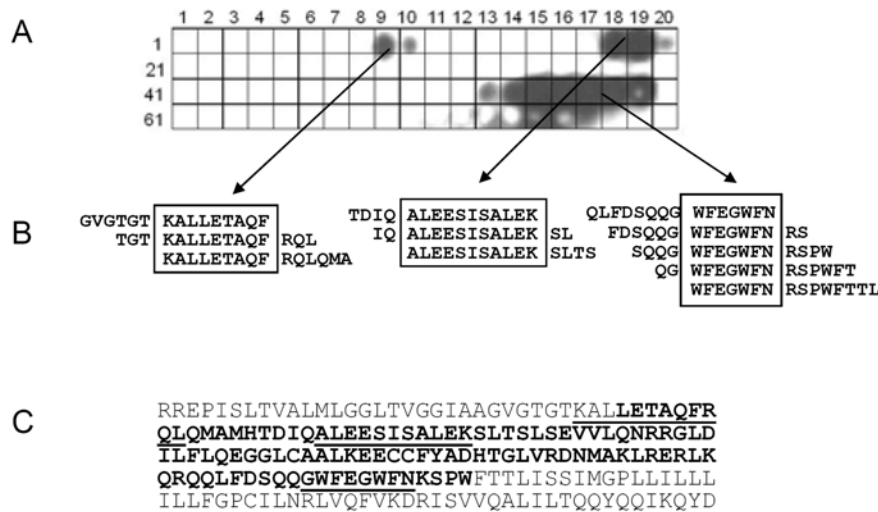
Figure 12 Epitope mapping using neutralising rat serum 15.3 specific for FeLV-A p15E. The result of the ECL dot blot using overlapping peptides is given in **A**, followed by the sequence alignment of the mapped peptides in **B**. In **C** the sequence of FeLV-A p15E is given, the sequence of the recombinant protein used for immunisation is printed in bold, and the epitopes are underlined.

The goat serum specific for FeLV p15E reacted with the epitopes E1a and E2a (Fig. 13) plus an epitope in the so-called immunosuppressive domain (Tacke et al., 2000). Interestingly, antibodies recognising this epitope were lost during affinity purification on p15E columns, but not when total IgG were isolated using protein G affinity chromatography.

The epitope E2b is closely related to the E2 epitope reported for a goat serum specific for PERV p15E (FEGWFN) (Fiebig et al., 2003) and rats immunised with this protein produced neutralising sera recognising epitopes corresponding to E2a and E2b (Fiebig et al., in preparation). Although, like the PERV p15E-specific serum, the FeLV p15E-specific serum detected only E1a and E2a (Fiebig et al., 2003), in both cases the goat sera had higher neutralising titres compared to the rat sera.
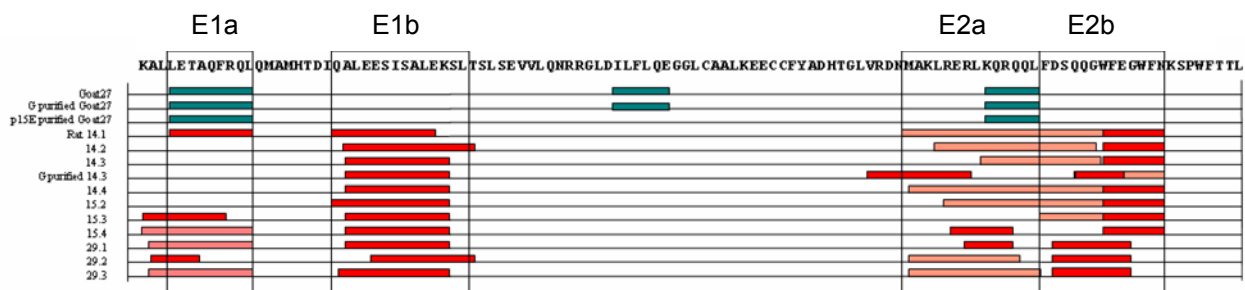
Figure 13 Results of the epitope mapping of different rat (marked in red) and goat (marked in green) antisera specific for p15E as well as of affinity purified IgG from two sera. The sequence of FeLV-A p15E is given in part, epitope regions are framed and indicated at the top.

Table 3 ELISA titres, neutralisation capacity and epitope mapping of FeLV-A p15E specific rat and goat sera

Antisera	ELISA titre[a]	Neutra-lisation[b]	Epitope mapping[c]			
			E1a	E1b	E2a	E2b
goat 27	$\geq 1\times 10^6$	99%	++	-	-	++
rat 14.1	1×10^6	51%	++	++	++	+
14.2	1×10^6	75%	-	++	++	+
14.3	1×10^6	94%	-	++	++	+
14.4	1×10^6	91%	-	++	++	+
15.2	1×10^6	81%	-	++	++	+
15.3	1×10^6	96%	++	++	++	+
15.4	1×10^6	80%	+	++	++	++
29.1	$\geq 2.5\times 10^5$	91%	+	++	++	++
29.2	$\geq 2.5\times 10^5$	98%	++	++	++	+
29.3	$\geq 2.5\times 10^5$	56%	+	++	++	+

[a] ELISA using recombinant p15E
[b] Inhibition of provirus integration at a serum dilution of 1:5, measured by real time PCR
[c] ++ strong detection, + weak detection, - no detection

3.1.5 p15E-specific antibodies recognise viral protein at the surface of FeLV-infected FEA cells

Antibodies purified from the goat serum by p15E-affinity chromatography antibodies were tested by immunofluorescence for binding to non-permeabilised FeLV-infected FEA feline embryonic fibroblast cells. The staining observed at the surface cell surface (Fig. 14B, C), suggests that the epitopes identified are accessible on the infected cell surface. To increase the quality of the pictures, unspecific cell fluorescence at 543nm was subtracted from FITC-specific signals at 488nm. Controls using uninfected cells or preimmune serum showed no staining (Fig. 14A).
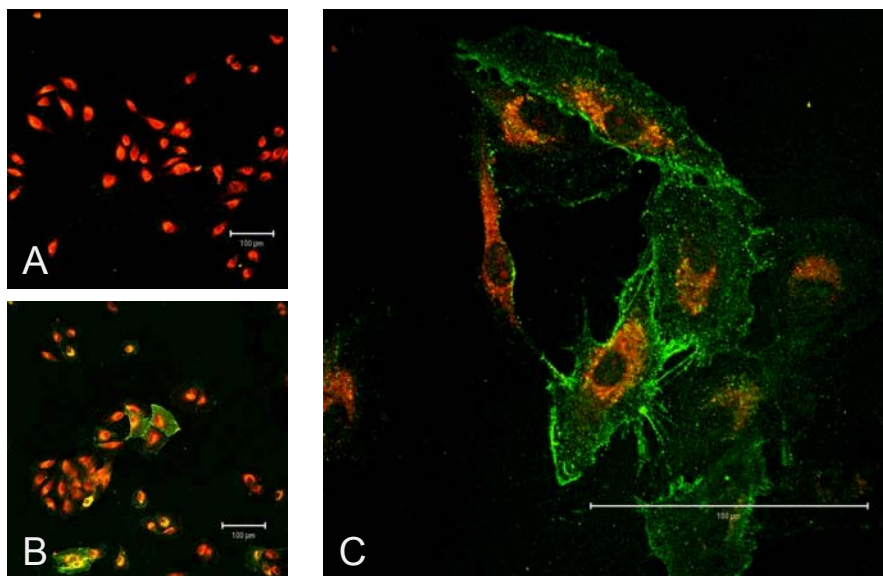
Figure 14 Indirect immuno fluorescence, visualised by confocal laser microscopy, using IgG purified by p15E affinity chromatography from goat serum specific for p15E and FITC conjugated anti-goat IgG Ab. FITC staining was measured at 488nm and unspecific fluorescence measured at 543nm was subtracted. Uninfected FEA cells are shown in (**A**), FeLV-A producing FEA cells are shown in (**B**) and (**C**). The white bars indicate 100µm.

3.2 Induction of neutralising antibodies against FeLV-A ΔISU p15E-antigen improvement

After the induction and characterisation of neutralising antibodies against FeLV-A p15E, the intention was to improve the antigen efficiency. Therefore the so-called immunosuppressive (isu) domain and a domain of the same length located in the C-terminal part in the p15E backbone was removed in order to maintain the localisation of previously identified epitope regions E1 and E2 (Fig. 15A, B). The isu-domain is highly conserved in all retroviruses and was removed because synthetic peptides corresponding to this domain inhibit lymphocyte proliferation and modulate cytokine production (Cianciolo et al., 1985; Denner 1998). These modifications also removed the sequences corresponding to two other peptides (J and K), which were shown in previous immunisation studies using different FeLV-derived peptides to induce enhancing antibodies (Fig. 15A) (Nick et al., 1990).

3.2.1 Characterisation of the antigen

The recombinant protein was termed FeLV-A ΔISU p15E and shown to have a size of 8.29kDa plus the additional 4kDa of the calmodulin binding peptide (CBP) at its N-terminus. The recombinant protein was shown to be insoluble and thus purification was performed by washing procedures of *E.coli* pellets containing the target protein eliminating hydrophilic proteins. Afterwards the protein was characterised by SDS-PAGE (Fig. 15C).
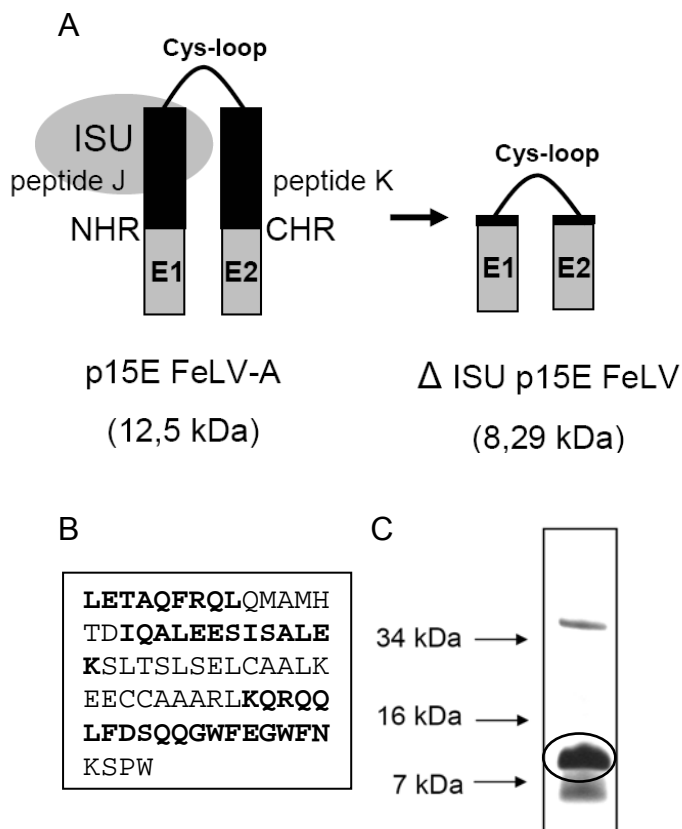
Figure 15 Characterisation of the FeLV-A ΔISU p15E protein. (**A**) Schematic presentation of the p15E ectodomain of FeLV and the modified ΔISU p15E protein. The localisation of the isu domain, the peptides J and K, the cystein loop as well the N- and C-helix regions (NHR, CHR) and the epitope regions (E1, E2) are indicated. (**B**), Amino acid sequence of FeLV-A ΔISU p15E. Identified epitope regions are printed in bold. (**C**) SDS-page analysis of semi-purified ΔISU p15E protein, framed in a circle.

3.2.2 Characterisation of binding and neutralising antibodies

Immunisation of 3 rats with FeLV-A ΔISU p15E resulted in the generation of antisera that recognised the recombinant protein in ELISA. Antibody titres ranged from 2.5×10^5 to 1×10^6 (Fig. 16A). The preimmune sera did not react in any assay. One of two sera was able to inhibit the infection of feline embryonic fibroblast cells by the FeLV-A Glasgow strain, whereas the preimmune serum had no such neutralising activity (Fig.16B). At a 1:16 dilution the rat serum 70.1 was able to inhibit infection by 70% with a neutralisation titre of $\geq$ 1:16.
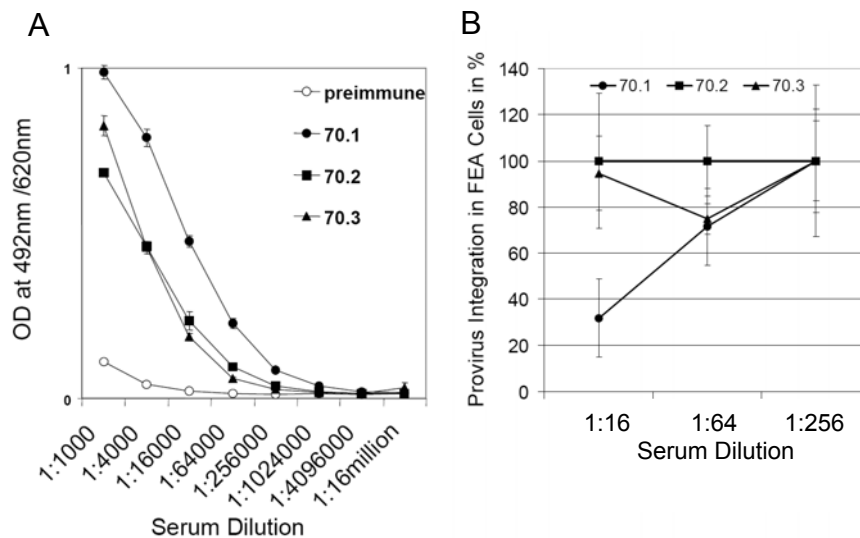
Figure 16 Characterisation of antisera from rats 70.1, 70.2 and 70.3. (A) Determination of the ELISA titres using the recombinant ectodomain of p15E as antigen. Immune sera and preimmune sera are indicated on the right. (B) Neutralisation of FeLV-A Glasgow strain at a serial dilution of antisera (1:16-1:256) indicated at the bottom. Infection was measured by real time PCR detecting provirus integration and indicated

3.2.3 Epitope Mapping

Linear 15-mer peptides (overlapping by 13 amino acids) corresponding to the entire FeLV-A p15E and bound covalently by the C-terminus to a cellulose membrane were tested for recognition by sera 70.1, 70.2 and 70.3. With rat serum 70.1 three epitopes were detected (Fig. 17): KALLETAQF, located at the N-terminal end, ALEESISALEK, located near the first epitope and KQRQQLF located N-terminal of p15E. These epitopes were previously designated E1a, E1b and E2a. Testing the sera of rats 70.2 and 70.3, two epitopes were detected located within the epitope regions E1 and E2. For both rat sera no epitope was found in the E2 region.
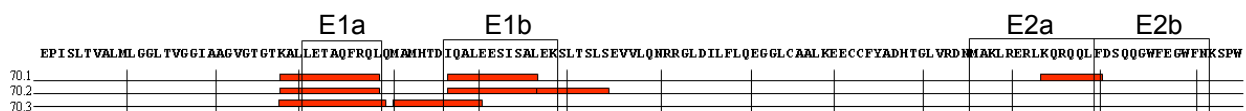
Figure 17 Results of the epitope mapping of rats 70.1, 70.2 and 70.3 as indicated at the left. The sequence of FeLV-A p15E is given in part, epitope regions are framed and indicated at the top.

3.3 Comparative studies between p15E and Leucogen induced neutralising antibodies in rats

In order to evaluate the FeLV-A p15E protein for the induction of neutralising antibodies in comparison to a commercial vaccine against FeLV, rats were immunised with p15E alone, with Leucogen, or with a combination of both. Leucogen contains the non-glycosylated surface envelope protein p45 of FeLV-A.

3.3.1 Binding antibodies specific for p15E and p45

When, in the first experiment, rats were immunised twice with 500µg p15E (group 50), 100µg Leucogen p45 (group 51) or a mixture of Leucogen and p15E (group 52), all sera showed strong ELISA reactivity specific for the corresponding antigen used for immunisation (Fig. 18A, B). Interestingly, the titre of binding antibodies specific for p45 was lower when Leucogen and p15E were injected simultaneously (group 52) in comparison to immunisation with Leucogen alone (group 51) (Fig. 18B). When this experiment was repeated immunising with 500µg p15E (group 55), 100µg p45 (group 56) and a mixture of both (group 54), ELISA titres of up to 4×10^6 were observed against the corresponding antigen used for immunisation (Fig 19A, B, D, E; Fig. 4, A, B; Tab. 4). Again, mixing of p45 and p15E decreased the antibody response to p45 in two cases, animals 54.1 and 54.2 when compared with animals that received only p45 (animal group 56) (Fig. 20; Tab. 4).
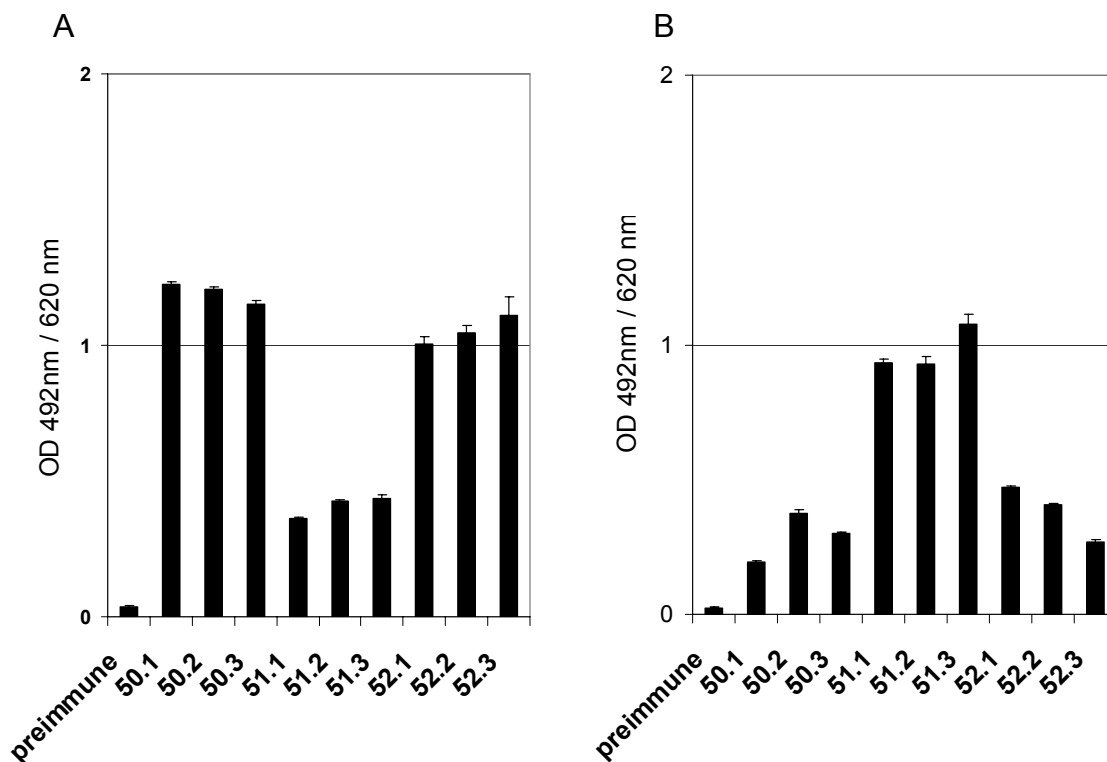
Figure 18 ELISA reactivity of rat antisera induced by immunisation with 500µg p15E alone (group 50), with 100µg p45 alone (group 51) and with a combination of both antigens (group 52). As antigen in the ELISA FeLV-A p15E (**A**) or Leucogen (**B**) were used.

There are three possible explanations for this result. First, the addition of p15E with its well-known immunosuppressive properties (Mathes, et al., 1979; Denner, 1987) may have reduced the production of antibodies specific for p45. Second, the addition of 500μg p15E to only 100μg p45 may have led to an antigenic dominance of p15E, and third, the addition of p15E to p45 may have led to interactions between domains of these two proteins, hiding epitopes of p45 from the immune system. *In vivo*, three transmembrane envelope proteins and three surface envelope proteins interact when building up the so-called knobs on the virus surface.

To investigate this further, Leucogen and p15E were injected at different sites (group 53) and the results were compared with the injection of a mixture at one site (group 54) (Fig. 20; Tab. 4) as had been performed in the first experiment (group 52) (Fig. 20; Tab. 4). Since in two cases (animals 54.1 and 54.2, Fig. 20; Tab. 4) the titre of the binding antibodies specific for p45 was reduced in comparison to all sera of group 53, it seems likely that the simultaneous injection of both antigens when injected into a single site was responsible for the decrease in antibody response.

To study the influence of the amount of p15E antigen on the induction of antibodies specific for p15E, injection of 500μg p15E (group 55) was compared with injection of 100μg p15E (group 57) (Fig. 21; Tab. 4). There was no obvious difference in the titres of binding antibodies as measured by ELISA. To investigate whether higher amounts of p15E in the antigen mixture has any influence on the antibody induction specific for p45, 100μg Leucogen were injected together with 500μg p15E (group 54) or together with 100μg p15E (group 60, Fig. 21; Tab. 4). There was no obvious effect of the increased p15E amounts on the production of binding antibodies specific for p45 or p15E.

55: 500μg p15E

56: 100μg p45

57: 100μg p15E
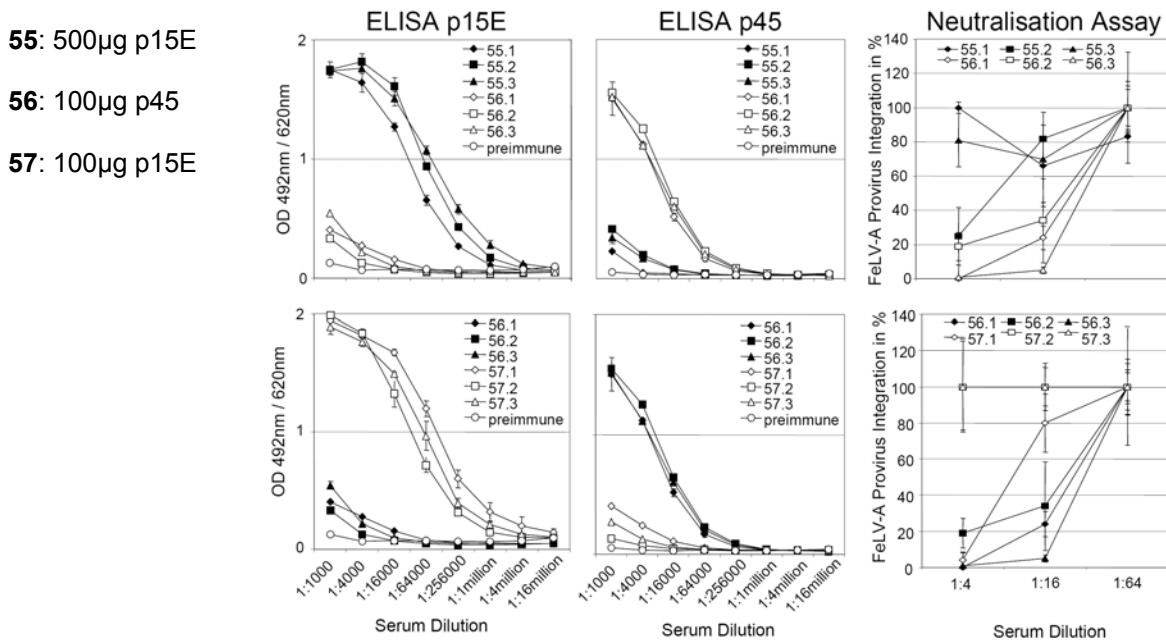
Figure 19 ELISA reactivity and neutralising activity of rat antisera induced by immunisation with 500μg p15E alone (group 55), with 100μg p15E alone (group 57) and with 100μg Leucogen alone (group 56). As antigen in the ELISA FeLV-A p15E or Leucogen were used.

53: 500µg p15E
  + 100µg p45
    seperate injecti

54: 500µg p15E
  + 100µg p45
    single injection

55: 500µg p15E

60: 100µg p15E
  +100µg p45
    single injection
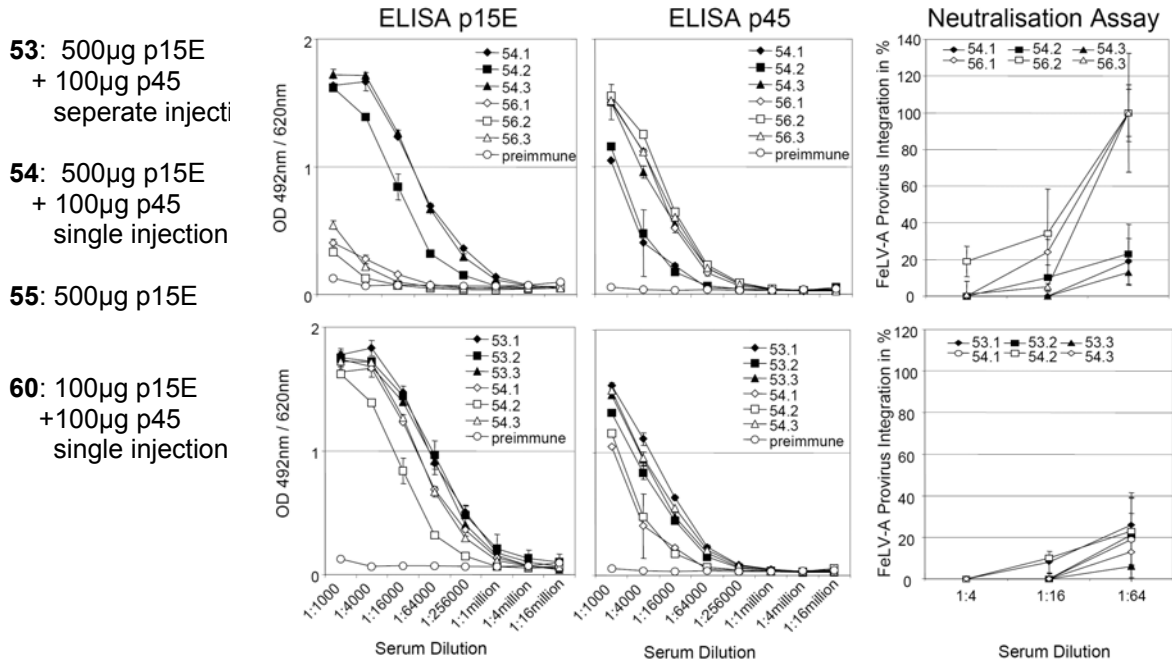
Figure 20 ELISA reactivity and neutralising activity of rat antisera induced by Immunisation with 500µg p15E in combination with 100µg Leucogen in a single injection site (group 54) or in two different injection sites (group 53) and with Leucogen alone (group 56). As antigen in the ELISA FeLV-A p15E or Leucogen were used.

54: 500µg p15E
  +100µg p45
    single injection

55: 500µg p15E

57: 100µg p15E

60: 100µg p15E
  +100µg p45
    single injection
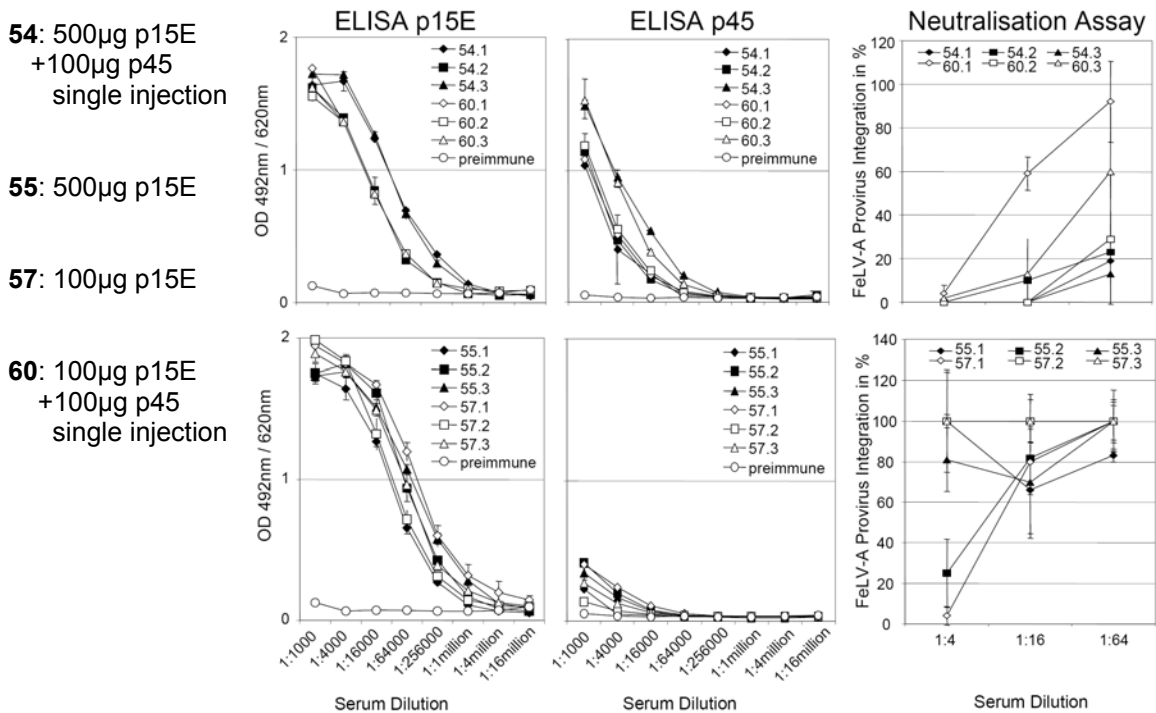
Figure 21 ELISA reactivity and neutralising of rat antisera induced by Immunisation with 500µg p15E in combination with 100µg Leucogen in a single injection site (group 54), with 100µg p15E in combination with 100µg Leucogen in a single injection site (group 60), with 500µg p15E alone (group 55) and with 100µg p15E alone (group 57). As antigen in the ELISA FeLVA p15E or Leucogen were used.

3.3.2 Induction and characterisation of neutralising antibodies

When tested for neutralising activity, 14 out of 18 antisera induced in the second experiment were able to inhibit the infection of FEA cells by FeLV-A with varying efficacies, whereas all preimmune sera had no such neutralising activity (Fig. 19; Fig. 20C; Fig. 21; Tab. 4). Sera from animals 55.1, 55.3, 57.2, and 57.3 did not show neutralising activity. To analyse the neutralisation efficacy, serial dilutions (1:4, 1:16 and 1:64) of the antisera were tested.

Antisera generated by immunising with 500µg p15E (group 55) showed neutralisation of FeLV-A ranging from nearly 0% (55.1 and 55.3) to 80% (55.2) at a 1:4 dilution (Fig. 19; Tab. 4). At the final serum dilution of 1:64 none of these sera had the ability to neutralise the virus. This confirmed previous data showing the induction of neutralising antibodies after immunising rats with 500µg p15E (chapter 3.1). Whereas in the previous immunisations Freund´s adjuvant was used, in this investigation Montanide® was employed as adjuvant. However due to the small number of animals, the influence of the adjuvant cannot be analysed.

When the amount of p15E antigen used for immunisation was reduced to 100µg (group 57), no reduction of the titre of neutralising antibodies was observed (Fig. 19), one antiserum (57.1) neutralised 90% of virus at a serum dilution of 1:4 but did not neutralise at 1:64. Two other antisera from animals of this group, 57.2 and 57.3 did not neutralise at any serum dilution.

Antisera obtained after immunisation with 100µg Leucogen p45 alone (group 56) neutralised at a range from 80% to 100% at a dilution of 1:4 and did not neutralise at 1:64. These data show that the neutralising capacity *in vitro* is much higher after immunisation with Leucogen when compared with immunisation with 500µg (Fig. 19; Tab. 4, group 56 versus 55) or 100µg p15E (Fig. 19; Tab. 4, group 56 versus 55,). When the neutralising capacity of the sera obtained after simultaneous immunisation with Leucogen and p15E (group 54) was compared with the neutralising capacity of sera obtained with Leucogen alone (group 56), better neutralisation was observed when both antigens were injected (Fig. 20). Interestingly, the increase in neutralising activity was associated with a decrease in the titre of binding antibodies specific for p45 (Fig. 20; Tab. 4).

However, when Leucogen and p15E were injected at different injection sites (group 53), the titres of binding antibodies specific for p45 was not reduced (Fig. 20; Tab. 4). This means that the titres of binding antibodies specific for p45 were comparable with titres in the sera from animals which received Leucogen alone (group 56, Fig. 20) and the neutralising capacity was as high as in the sera from animals which received simultaneously Leucogen and p15E at a single injection site (group 54, Fig. 20; Tab. 4). In the case that 500µg p15E and 100µg p45 were separately injected at different injection sites (group 53), neutralisation efficacies ranging from 100% at a serum dilution of 1:4 to about 75% or more at a serum dilution of 1:64 were observed (Fig. 20; Tab. 4). The antisera obtained after immunisation using a single injection site for 500µg p15E and 100µg p45 (group 54) showed neutralisation efficacy ranging from 100% at a serum dilution of 1:4 to about 80% or more at a serum dilution of 1:64 (Fig. 20; Tab. 4). This indicates that the titre of neutralising antibodies is not affected

by the addition of p15E to p45 despite the finding that the titre of binding antibodies specific for p45 was reduced in two cases (Fig. 20; Tab. 4).

To evaluate the influence of the amount of p15E on the antibody response against p45, animals were immunised with Leucogen either together with 500μg (group 54) or together with only 100μg p15E (group 60) (Fig. 21, Tab. 4). The binding antibody response specific for p15E was slightly lower in the group that received only 100μg p15E (Fig. 21). As already mentioned above, the binding antibody response specific for p45 was identical in both groups (Fig. 21). In contrast, the neutralising capacity was significantly higher in sera from animals that received 500μg p15E (Fig. 21; Tab. 4), indicating that higher amounts of p15E induced more neutralising antibodies in connection with Leucogen. When 500μg (group 55) or 100μg p15E (group 57) were applied without Leucogen, no differences in the binding antibody response specific for p15E and in the neutralising capacity (Fig. 21; Tab. 4) were observed.

3.3.3 Epitope mapping

An epitope mapping of the induced sera was performed using linear 15-mer peptides (overlapping by 13 amino acids) corresponding to the entire p15E of FeLV-A, bound covalently by the C-terminus to a cellulose membrane. When sera from rats immunised with recombinant p15E alone (groups 55, 57) were analysed, four main epitope regions were found, two at the N terminus and two at the C terminus of the ectodomain (Fig. 22; Tab. 4). These findings confirmed our previous studies, in which the same epitope regions were identified and were designated E1a (LETAQFRQL) and E1b (IQALEESISALEK) as well as E2a (KQRQQL) and E2b (FDSQQGWFEGWFN). In contrast to the previous study (chapter 3.1), the E2a epitope was better defined (KQRQQLF instead of MAKLRERLKQRQQL). These four epitopes were identified, regardless of whether 500 μg (group 55) or 100μg (group 57) of p15E were applied (Fig. 22; Tab. 4). Interestingly, none of the non-neutralising sera recognised the E1 epitopes, suggesting that this epitope is essential for neutralisation. The same epitopes were identified when rats were immunised with p15E and Leucogen (animal groups 53, 54 and 60), indicating that Leucogen did not change the recognition of the epitopes by the immune system (Fig. 22; Tab. 4). It was also shown that the adjuvant does not influence the recognition of the epitopes: regardless of whether Freund´s adjuvant, Montanide[®] (groups 55 and 57) or the adjuvant contained in the Leucogen preparation (groups 53, 54 and 60) were used, the same epitopes were recognised. As expected, no p15E specific epitopes were detected when sera were tested from rats immunised with Leucogen alone (group 56), indicating that the Leucogen vaccine does not contain parts of the transmembrane envelope protein.
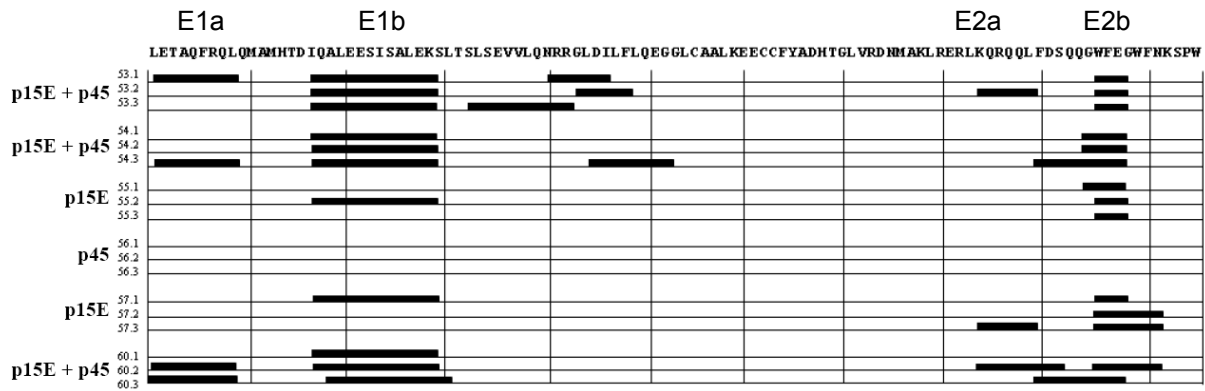
Figure 22 Summary of the specific epitope mapping of all antisera obtained after immunisation with p15E, Leucogen or both, p15E and Leucogen. The sequence of the FeLV-A p15E ectodomain and the animal number and the antigen(s) used for immunisation are given, epitopes are framed.

Table 4 Titres of binding and neutralising antibodies and epitope mapping after immunisation with Leucogen, p15E or both antigens.

Group	Rat	Antigen(µg)		Application site for combined immunisations	Adjuvant[a]	Titres p15E (ELISA)	Titres p45 (ELISA)	Neutralisation Titre	Epitope mapping			
		p15E	Leucogen®						E1a	E1b	E2a	E2b
53	53.1				LA and Montanide	1×10^6	6.4×10^4	1:64	+	+	-	+
	53.2	500	100	Two separate		1×10^6	6.4×10^4	1:64	-	+	+	+
	53.3					1×10^6	6.4×10^4	1:64	-	+	-	+
54	54.1					1×10^6	1.6×10^4	1:64	-	+	-	+
	54.2	500	100	Single	LA	2.56×10^5	1.6×10^4	1:64	-	+	-	+
	54.3					1×10^6	6.4×10^4	1:64	+	+	-	+
55	55.1				Montanide	1×10^6	-	0	-	-	-	+
	55.2	500	-	-		4×10^6	-	1:16	-	+	-	+
	55.3					4×10^6	-	0	-	-	-	+
56	56.1					-	6.4×10^4	1:16	-	-	-	-
	56.2	-	100	-	LA	-	6.4×10^4	1:16	-	-	-	-
	56.3					-	6.4×10^4	1:16	-	-	-	-
57	57.1				Montanide	4×10^6	-	1:16	-	+	-	+
	57.2	100	-	-		1×10^6	-	0	-	-	-	+
	57.3					4×10^6	-	0	-	-	+	+
60	60.1					2.56×10^5	1.6×10^4	1:16	-	+	-	-
	60.2	100	100	Single	LA	2.56×10^5	1.6×10^4	1:64	+	+	+	+
	60.3					2.56×10^5	6.4×10^4	1:64	+	+	-	+

[a]LA – Leucogen adjuvant containing Quil-A and aluminium hydroxide, Montanide ISA 720

3.4 Induction of neutralising antibodies against p15E in cats

Since it was shown that immunisation with FeLV-A p15E results in the induction of neutralising antibodies in different species (chapter 3.1 and 3.2) finally it should be determined if this is also the case in its natural host. Therefore three cats were immunised with p15E and the humoral immune response was characterised and compared to naturally FeLV-infected housecats.

3.4.1 Induction of binding antibodies specific for p15E in cats

In Western blot analyses, antisera from all three cats immunised with 500μg p15E (#14, #34, and #44) specifically detected the recombinant p15E protein at a size of 15kDa, while the preimmune sera did not react (Fig. 23A). When sera from FeLV-infected housecats were tested in the same assay, 44 of 75 sera (58.6%) also specifically detected p15E, four of which are shown in Fig. 1a. FeLV infection had been diagnosed in all animals using a commercial antigen detection assay. These data indicate that immunised and infected animals are able to produce antibodies specific for p15E.
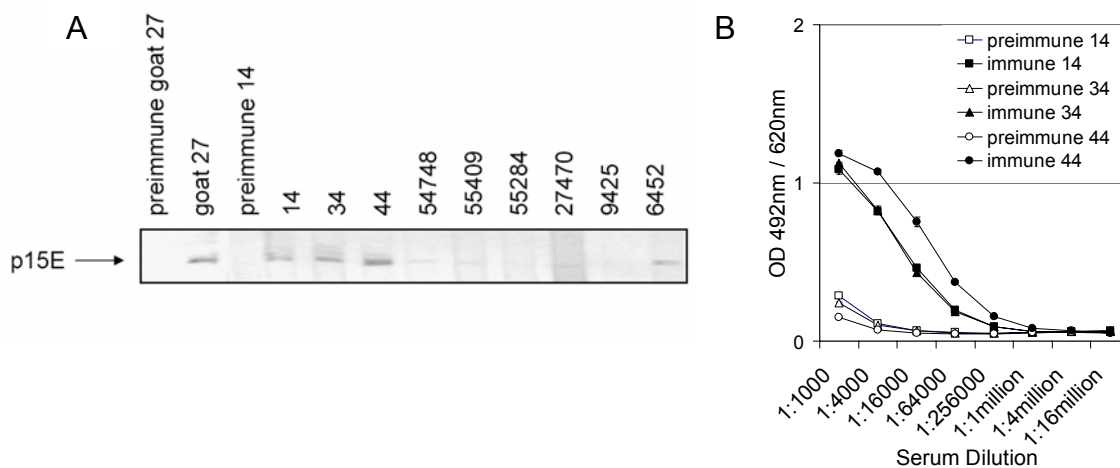
Figure 23 (**A**) Western blot analysis of cat antisera following immunisation with FeLV-A p15E (14, 34 and 44) and of sera from FeLV-infected cats (54748-6452). The same recombinant p15E used for immunisation was used as antigen. Lane 1 shows the preimmune serum of goat 27, lane 2 the corresponding immune serum. Only one preimmune cat serum is shown (#14, lane 3) (**B**) ELISA reactivity of cat sera induced by immunisation with 500μg p15E in comparison with the corresponding preimmune sera. Recombinant p15E was used as antigen.

All three immune sera, but not the preimmune sera, also reacted strongly in ELISA using recombinant p15E as antigen (Fig. 23B). After the boost immunisation, the titre of binding antibodies increased markedly (Tab. 5). Sera from cat 44 showed the highest titres of binding antibodies in this group (2.56 x10^5 after the first immunisation and 1x10^6 after the boost). The sera from cats 14 and 34 showed titres of 6.4x10^4 that increased to 2.56 x10^5 after the boost immunisation. In contrast, sera obtained from FeLV-infected housecats had only titres between 1x10^3 and 4x10^3 (Tab. 5).

3.4.2 Induction of neutralising antibodies specific for p15E in cats

Neutralisation of FeLV-A strain Glasgow infection of feline embryonic fibroblast cells was measured using four-fold serial dilutions (from 1:16 to 1:16384) of the sera. All sera taken after the first immunisation had titres of 1:256 (Fig. 24) whereas no preimmune sera showed neutralising activity. Similar to the titres of binding antibodies, the titres of neutralising antibodies increased after the booster immunisation in two animals (cats 14 and 34) up to 1:1024 (Tab. 5) although the titre of neutralising antibodies in the serum of cat 44 did not increase.
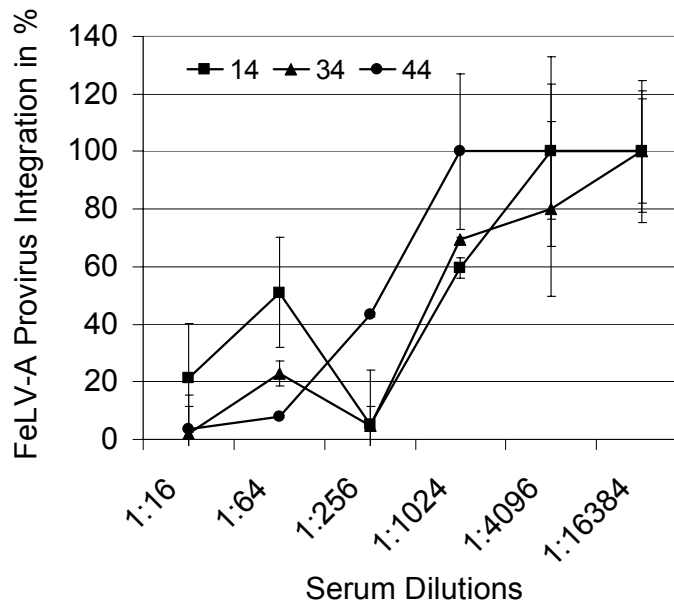
Figure 24 Neutralising activity of cat antisera after the second immunisation with 500µg p15E. Infection was measured as provirus integration by real-time PCR. Percent of provirus integration was obtained by comparing antisera with the corresponding preimmune sera.

3.4.3 Epitope mapping

To identify the epitopes recognised by the immune sera, epitope mapping was performed using linear 15-mer peptides overlapping by 13 amino acids corresponding to the entire FeLV-A p15E and bound covalently by the C-terminus to a cellulose membrane. Four major epitopes were identified (Fig. 25) using sera from the cats immunised with p15E (14, 34, and 44). The first epitope, KALLETAQF, is nearly identical to an epitope identified by immunising a goat with FeLV p15E and to a consensus epitope (LETAQFRQL) recognised by sera from 8 rats immunised with the same antigen. This epitope group was designated E1a. The second epitope (ALEESISALEK, E1b) was also recognised by all 8 rat sera but not by the goat serum. The third epitope is located in the immunosuppressive domain, LQNRRGLDILFLQEGGL, which is highly conserved amongst all retroviruses (Denner et al., 1994). Synthetic peptides corresponding to this domain inhibit lymphocyte proliferations and modulate

cytokine production (Denner 1998). This epitope in the immunosuppressive domain was also recognised by the goat serum, but not by any of the rat sera. The fourth epitope, MAKLRERLKQRQQLF, corresponds to an epitope E2a, recognised both by the goat serum and by 7 of 8 rat sera. Similar to the goat serum the cat sera did not recognise an epitope recognised by all rat sera and designated E2b (FDSQQGWFEGWFN). Therefore, the cat sera bind to main epitopes already described following immunisation of rats and goats. These data support the existence of main target epitopes after immunisation with p15E and minor species-specific differences.

Figure 25 Epitope mapping using sera from immunised and FeLV-infected cats. Summary of the epitopes identified recognised by each serum. Sequences corresponding to the recombinant p15E of FeLV-A, strain Glasgow 1, and the corresponding sequence of an endogenous p15E are given at the top. Cats 14, 34 and 44 were immunised with p15E while the others are representative of FeLV-infected cats. Strong epitopes are marked in black, weak epitopes in grey. Common 2 groups of epitopes are framed (E1a, E1b, E2a, E2b). In addition, epitopes recognised by the serum from goat 27, immunised with p15E, and consensus epitopes recognised by 8 rats immunised with p15E are shown (hatched)6. For comparison, the C-terminal part of the HIV-1 transmembrane envelope protein gp41 and the localisation of epitopes recognised by two monoclonal antibodies (mAb) broadly neutralising HIV-1, (2F5 and 4E10) are shown (framed) (see appendix 7.1 for detail).

3.4.4 Sequences homologous to the epitopes are present in endogenous retroviruses

When the sequence of the FeLV-A p15E used for immunisation was compared with that of the endogenous feline retrovirus CFE-6 (NCBI accession no. gi:74706), sequence homologies were identified in the epitope domains (Fig. 25). Therefore, despite such sequences being present in the genomes of the immunised cats, binding and neutralising antibodies specific for these domains were induced.

3.4.5 p15E-specific antibodies recognise viral protein at the surface of FeLV-infected cells

To elucidate the possible mechanisms of neutralisation, the localisation on the cell surface of the epitopes recognised by the p15E-specific sera was analysed by immunofluorescence using non-permeabilised FeLV-infected FEA feline embryonic fibroblast cells. Uninfected cells were not recognised by cat sera #14, #34, and #44 were used (Fig. 26A). However, all three bound to the cell

surface (Fig. 26B) whereas the corresponding preimmune sera did not. To increase picture quality, unspecific cell fluorescence at 543nm was subtracted from FITC-specific signal at 488nm. This binding of immune sera to the cell surface indicates that the epitopes identified are accessible to FeLV on the surface of infected cells.
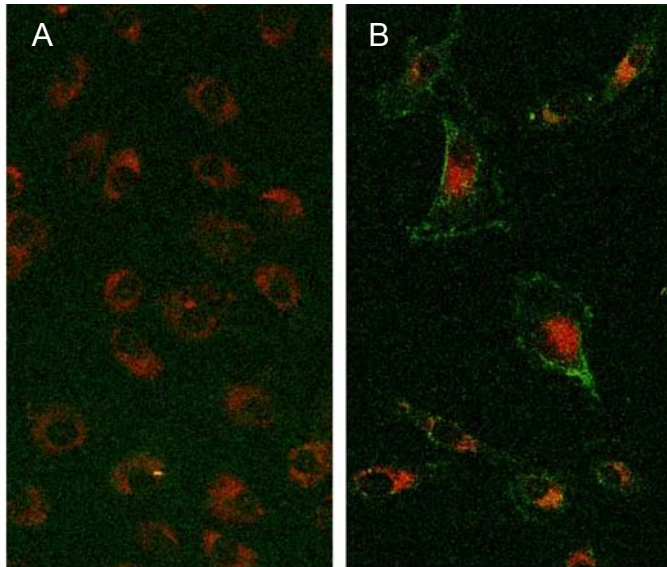
Figure 26 Indirect immunofluorescence, visualised by confocal laser microscopy, using antiserum from cat 44 (immunised with p15E) and FITC-conjugated anti-cat IgG. FITC staining was measured at 488nm and unspecific fluorescence measured at 543nm was subtracted. (**A**) Uninfected FEA cells, (**B**) FeLV-A producing FEA cells.

3.4.6 Neutralising antibodies in the sera of FeLV-infected cats

To compare neutralising antibody responses in FeLV-infected cats with those of p15E-immunised animals, sera from infected housecats were analysed. As described above, 44 of 75 sera investigated showed antibodies specific for p15E by Western blot analysis (Fig. 23A) and the titres of p15E-specific antibodies in ELISA ranged from $\leq 1 \times 10^3$ to 4×10^3 (Tab. 5). Neutralising titres of these sera were found to be between 0 and 1:256 (Tab. 5), although it must be kept in mind that in the infected cat neutralising antibodies might also be directed against other viral proteins such as gp70. Epitope mapping using overlapping peptides spanning the entire p15E (Fig. 25) was carried out. Serum from cat 9425 only recognised the epitope E2a, while serum from cat 6452 recognised an epitope located outside E1a as well as E1b and E2a, and serum from cat 27047 recognised the epitopes E1a and E1b weakly, but E2a more strongly. Cat 27047 had initially been immunised with Leucogen containing the nonglycosylated surface envelope protein p45 but became infected despite this immunisation. Sera from cats 54748 and 55409 (Fig. 25) weakly detected epitopes E2a and E2b but none of these epitopes were recognised by serum from cat 55284, despite this cat being infected and having low titre neutralising antibodies.

Table 5 Characterisation of sera from immunised and FeLV-infected cats. Titres shown in brackets were obtained after the first immunisation.

Serum	Immunisation	FeLV diagnosis	Western blotting p15E	ELISA titre[a]	Neutralisation titre[b]	Epitope mapping[c]			
						E1a	E1b	E2a	E2b
14	p15E	-	+	$(6,4 \times 10^4)$ $2,56 \times 10^5$	(1:256) 1:1024	++	+	++	-
34	p15E	-	+	$(6,4 \times 10^4)$ $2,56 \times 10^5$	(1:256) 1:1024	++	+	++	-
44	p15E	-	+	$(2,56 \times 10^5)$ 1×10^6	(1:256) 1:256	++	+	++	-
9425	none	+	-	$\leq 1 \times 10^3$	1:16	-	-	-/+	-
6452	none	+	+	4×10^3	1:256	-	++	++	-
27470	various vaccines	+	-/+	4×10^3	1:256	+	+	-/+	-
54748	none	+	+	4×10^3	0	-	-	+	+
55409	none	+	-/+	4×10^3	1:64	-	-	+	+
55284	none	+	-	$\leq 1 \times 10^3$	1:64	-	-	-	-

a): ELISA using recombinant p15E

b): Inhibition of provirus integration measured by real time PCR in comparison to preimmune sera

C): ++ strong detection, + weak detection, - no detection

3.5 Challenge studies in p15E and Leucogen immunised cats

Since the induction of neutralising antibodies by immunisation with the FeLV-A p15E protein was shown in cats (chapter 3.4) it should be analysed here if a p15E immunisation can induce a protective immunity *in vivo*. Challenge studies in cats were performed preliminary immunised with p15E, with Leucogen or with a combination of both. Prior to the virus challenge the immune status of the immunised cats was determined. Integrated in this study were cats 14, 34 and 44 previously shown to have neutralising antibodies after immunisation with p15E (chapter 3.4).

3.5.1 Immune response of the vaccinated and control animals before challenge

The antibody response of cats which had been immunised twice with the ectodomain of p15E, with Leucogen or with a combination of both, and of non-immunised control cats, was investigated four weeks after the last boost and 16 weeks later, three days before challenge. Four weeks after immunisation with p15E alone or in combination with Leucogen, binding antibodies specific for p15E were detected in all animals. In an ELISA using the ectodomain of p15E as antigen, titres of binding antibodies ranging from 2.56×10^5 to 4×10^6 were found (Fig. 27A). Cat 53.3 showed the highest titre. As expected, no antibodies specific for p15E were found in the sera from cats immunised with Leucogen alone. The sera from cats immunised with Leucogen alone reacted with p45 and showed ELISA titres ranging from 6.4×10^4 to 2.56×10^5 (Fig. 27B). The serum from cat 32.3 showed the highest titre. As expected, none of the sera from cats immunised with p15E showed antibodies specific for p45 (Fig. 27B). After immunisation with a combination of p15E and Leucogen (animals 50.3 and 51.3) ELISA titres of 1×10^6 for p15E-specific antibodies (Fig. 27A) and of 2.56×10^5 of p45-specific antibodies was found. The titres of binding antibodies to each antigen, p15E or p45, were in the same range as after immunisation with each antigen alone, indicating that each does not influence the response to the other.

The neutralising capacity of the sera from immunised cats was determined in a neutralisation assay using FeLV-A and measuring provirus integration by real time PCR. The neutralising capacity was expressed as percent neutralisation in comparison with the corresponding preimmune serum (see experimental procedures). The sera were used at a dilution 1:100. Four weeks after the boost immunisation all but one serum (51.3 not tested) showed neutralising activity (Fig. 27C) and the neutralising activity of sera after immunisation with p15E was comparable with that of sera after immunisation with Leucogen. Sera from three animals immunised with p15E (cats 34, 44 and 53.3) showed high neutralising activity (up to 92%), while the other three had low or medium activity. Sera obtained after immunisation with Leucogen alone showed a neutralising activity between 47% (cat 54) and 91% (cat 32.3). The serum from cat 50.3, immunised with a combination of p15E and Leucogen showed a neutralising activity of 90%, (while serum from cat 51.3 as an exception did not show any neutralising activity) (Fig. 27C).
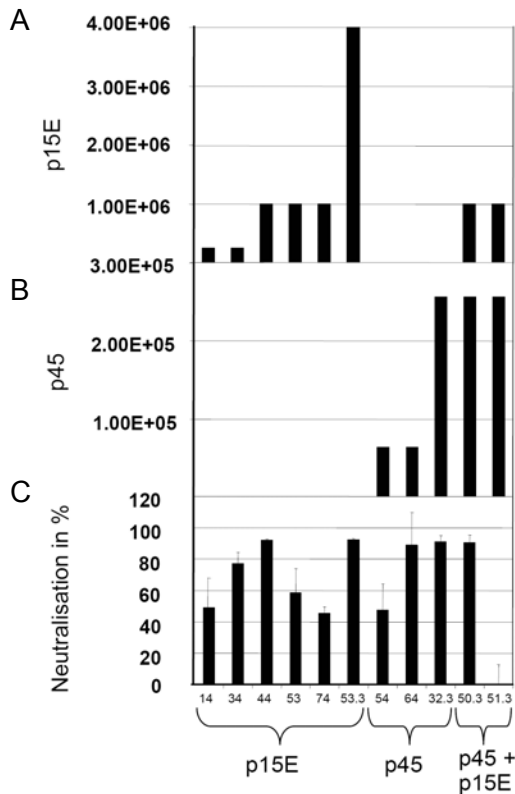
Figure 27 Titres of binding antibodies against p15E (**A**) and p45 (**B**) determined in an ELISA with a four fold serial dilution of antisera compared to corresponding preimmmune sera. (**C**) Neutralising activity in percentage obtained for sera from immunisation with p15E, Leucogen or with a combination of both as shown by indicated brackets. The neutralising activity was determined in relative to corresponding preimmmune sera.

To study how long these neutralising antibody titres persisted, sera were tested again after 4 months, which is 3 days before challenge. It was interesting to see a nearly parallel decline of the neutralising titres of sera in this short time (with the exception of the serum from cat 51.3, which showed an increased activity) (Fig. 28). Nevertheless, two sera from cats immunised with p15E alone (cat 14, 34) or in combination with Leucogen (cat 50.3) still showed significant neutralising activity, although it was reduced about 2-fold in comparison to the activity 4 months earlier. It is important to note that all antisera still showing neutralising activity at that time were derived from animals immunised with p15E, either alone or in combination with Leucogen.
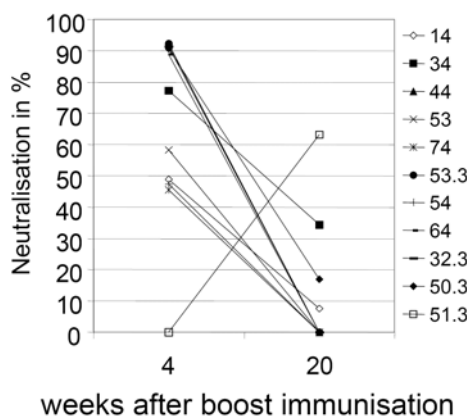
Figure 28 Comparison of neutralising antibodies 4 weeks and 20 weeks after immunisation corresponding to three days before the virus challenge. Shown is the percentage of neutralising activity for sera ontained from immunisation with p15E, Leucogen or with a combination of both as shown by indicated brackets. The neutral-lising activity was determined in relative to corresponding preimmmune sera.

3.5.2 Protection was induced by the TM protein p15E as well as by Leucogen

Protection from FeLV challenge is indicated by a failure of the establishment of a persistent viraemia in vaccinated cats. The measurement of the FeLV p27 antigen in the peripheral blood is the most common method for the diagnosis of FeLV viraemia. Cats are considered FeLV negative if the OD value in the p27 ELISA is below a pre-defined cut off value. The percentage of p27 antigen load is usually determined by setting the positive control as 100%. For this analysis a commercial assay was used (see experimental procedures).

The outcome of the challenge is summarised in Figure 3. None of the cats showed p27 antigen in the blood when tested 3 days before and 10 days past challenge (Fig. 29A). However, 30 days after the challenge, p27 antigen was detected in the sera of all non-immunised control cats (16.4, 22.4, 35.3), with activities ranging from 47% to 92% of the control (Fig. 29B). Therefore all of these animals were considered to be FeLV positive. The non-immunised animals also showed the highest p27 antigen levels (values between 82% and 125%) 80 days after the challenge. Thereafter the level decreased to values between 18% and 46%. Analysing the animals immunised with p15E alone, two cats (14 and 74) were p27 positive and four cats (44, 34, 53, 53.3) were negative 30 days after challenge. The p27 antigen detected in the peripheral blood of cat 14 was in the same range (98%) as in the blood of non-immunised animals of the control group, whereas cat 74 showed a significantly lower p27 antigen level (11%). Sixty days after challenge, four cats were found to be positive. In addition to cats 14 and 74 that were already positive at day 30, cat 44 with 18% and cat 53 with 94% p27 antigen were also found positive. The level of p27 antigen in the serum from cat 44 was significantly lower than those from all other cats that were positive. At day 80 and day 100 after challenge cats 14, 53 and 74 were FeLV positive while cats 34, 44 and 53.3 remained negative. It appears that cat 44 was transiently viraemic while cat 53 became viraemic later; this interpretation is consistent with the development of virus neutralising antibodies in cat 44 and a decline in antibody titre in cat 53 by day 100. In the cats immunised with Leucogen alone or in combination with p15E (Leucogen: 54, 64, 32.3; combination: 50.3, 51.3) p27 antigen was never detected in the blood. In addition, p27 antigen was never detected in cats 34, 44 and 53.3 immunised with p15E. Therefore, according to the commercial ELISA used, all 5 animals immunised with Leucogen, either alone or together with p15E and three of the 6 animals immunised with p15E alone were protected.
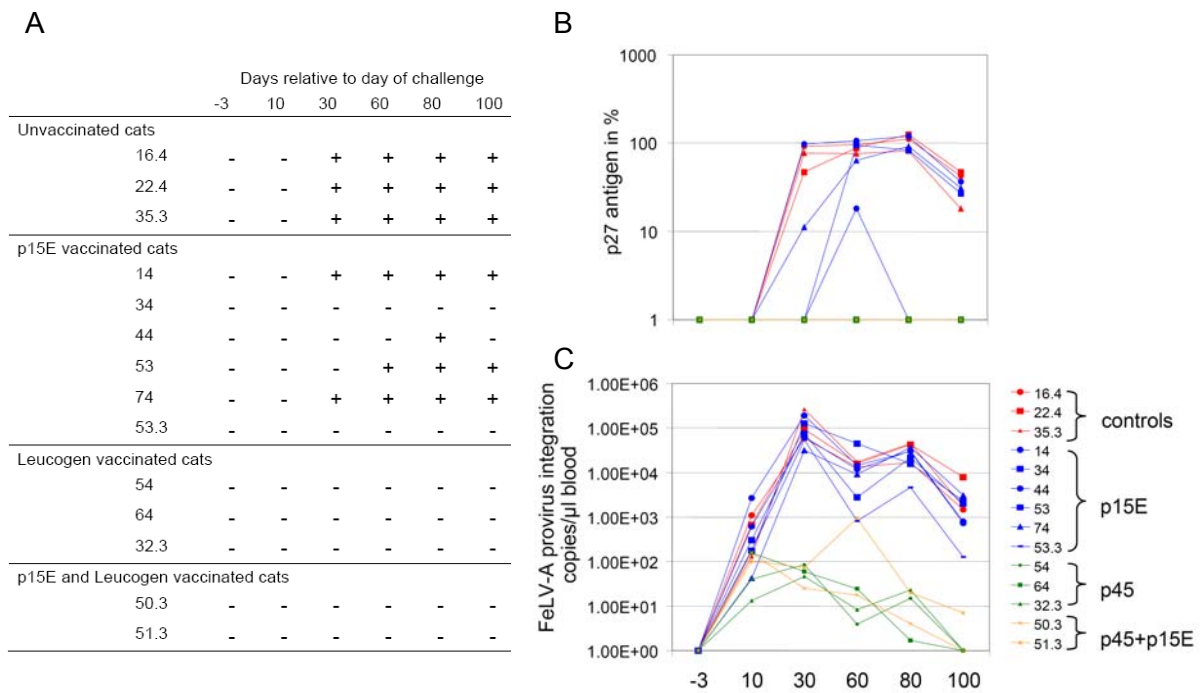
Figure 29 Response of cats to challenge with FeLV (**A**) Summary of presence (+) or absence (-) of p27 antigen from blood samples. (**B**) Kinetics of FeLV provirus integration in copies/µl blood measured in non-immunised control animals (red), in animals immunised with p15E (blue), with Leucogen alone (green) or in combination with p15E (yellow). The FeLV-A provirus integration was determined using a virus-specific real time PCR. (**C**) Kinetics of FeLV-A p27 antigen detection in blood cells (in % relative to FeLV-A sera from persistently infected control cats) of the animals described in A. FeLV-A p27 antigen measurement was performed in an ELISA and values below a predefined cut off were considered as FeLV negative.

3.5.3 Immunisation with Leucogen, with the TM protein p15E or with a combination of both did not result in sterilising immunity

To characterise the level of protection in more detail, the provirus load was analysed in peripheral blood cells. For this, a real time PCR specific for FeLV was developed and the provirus integration was measured as copies/µl blood. No cat showed provirus integration in the blood tested before challenge, confirming that uninfected animals were used and that the primers used in the PCR did not detect feline endogenous retroviruses. In the blood cells of the non-immunised control animals (16.4, 22.4, 35.3) provirus integration was observed starting with day 10 after challenge. At that time, between $1.08x10^2$ and $1.26x10^2$ copies/µl blood were observed, increasing up to $5.82x10^4$ and $2.66x10^5$ copies/µl blood at day 30 after challenge. This level decreased to below $1x10^4$ copies/µl blood at day 100 after challenge.

In all six animals immunised with p15E alone (cats 14, 44, 34, 53, 74, 53.3), a similar cell associated virus load was found in the non-immunised control group. Only cat 53.3 (the animal with the highest titre of binding and neutralising antibodies) showed significantly lower provirus load starting at day 60 after challenge until the end of study. At day 60 and at day 100 cat 53.3 showed a provirus load below $1x10^3$ copies/µl blood. In addition, cats 14 and 44 also showed a reduced level of provirus integration

in comparison to the cell-associated virus load in non-immunised control animals 100 days after the challenge (Fig. 29B).

In contrast to the animals of the non-immunised control group and to the animals immunised with p15E alone, low levels of provirus integration were observed in the blood of animals immunised with Leucogen alone or in combination with p15E (Leucogen: 54, 64, 32.3; combination: 50.3, 51.3). Starting with day 10 after challenge the level of provirus integration ranged between 1.32×10^1 and 1.57×10^2 copies/µl blood decreasing to 7.05 copies/µl blood (animal 50.3) or to a complete clearance of provirus at day 100 after the challenge. There was no detectable difference in the provirus load between animals immunised with Leucogen alone or with the combination. In both groups provirus load was about 100 fold lower when compared with the non-immunised control animals or in the animals immunised with p15E alone beginning with day 30 after challenge (Fig. 29B).

3.5.4 Provirus load and virus load correlate inversely with neutralising antibodies

To analyse whether neutralising antibodies represent a correlate for protection, the relationship between provirus load in copies/µl blood and the p27 antigen load in percentage on one hand and the neutralising capacity of the serum on the other was investigated.

Sera from all cats including the non-immunised animals had neutralising antibodies beginning on day 10 (except animal 44 immunised with p15E) (Fig. 30). Some of the immunised cats, however, already had immunisation-induced neutralising antibodies at the day of challenge. Such antibodies were found in two of six animals immunised with p15E alone and in both animals immunised with p15E and Leucogen, but in none of the animals immunised with Leucogen alone. The kinetics of the titres of neutralising antibodies showed two maxima in most animals. In the case of the non-immunised animals (where the neutralising antibodies were induced solely by infection) the titre decreased finally to zero at day 100. In all other animals the neutralising activity increased up to 100%. However, correlating with the p27 antigen load great differences were observed. Animals with high p27 antigen load had neutralising activities below 100%, whereas in all animals where p27 antigen was not detected, the neutralising activity always reached 100% at day 100 (except cat 32.3). When comparing the animals immunised with Leucogen alone with the animals immunised with the combination of Leucogen and p15E, only one important difference was observed: in both animals immunised with p15E, neutralising antibodies existed already at the day of challenge (obviously induced by immunisation with p15E), whereas animals immunised with Leucogen did not have neutralising antibodies. Taken together, these data show that at the day of challenge no neutralising antibodies were detected in animals immunised with Leucogen, although immediately after immunisation these antibodies could be detected. Most important, we clearly show that there is a strong inverse correlation between the neutralising activity and the p27 antigen load. This may indicate that the neutralising antibodies suppress the virus load or that the developing persistent viraemia inhibits the production of FeLV-specific antibodies.

Unvaccinated cats
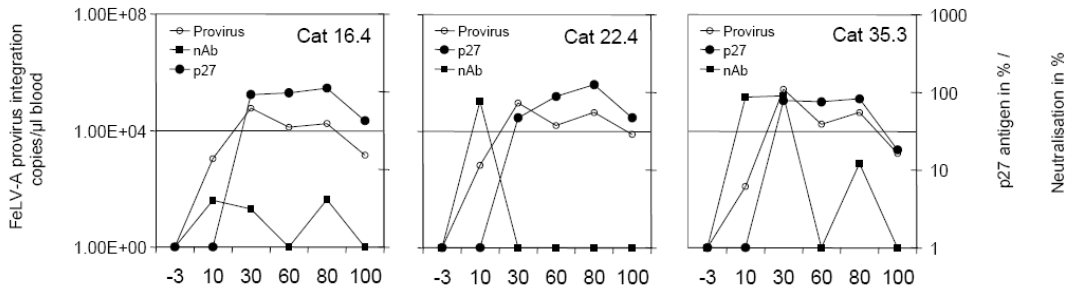
p15E vaccinated cats
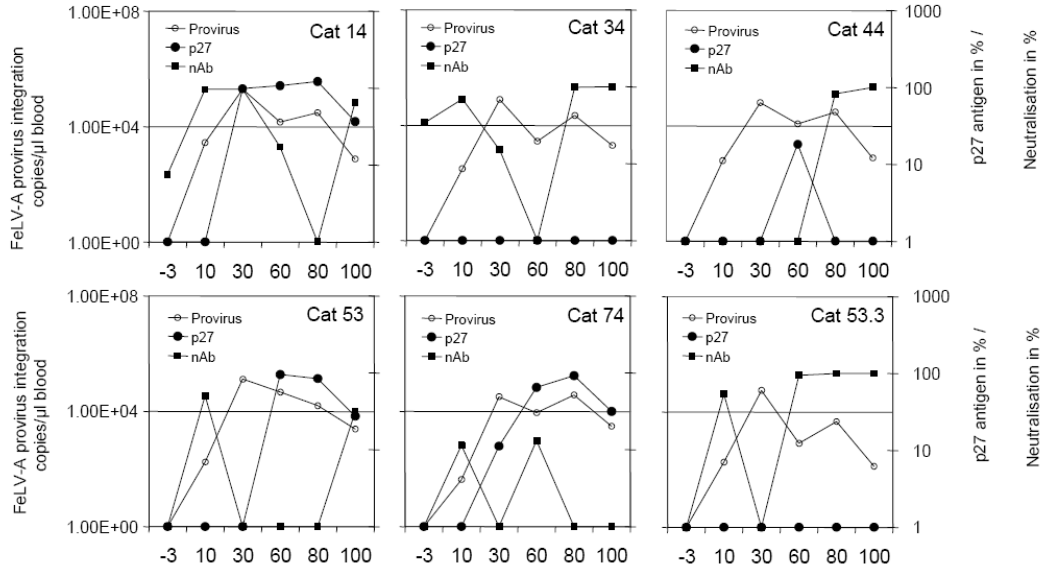
Leucogen vaccinated cats
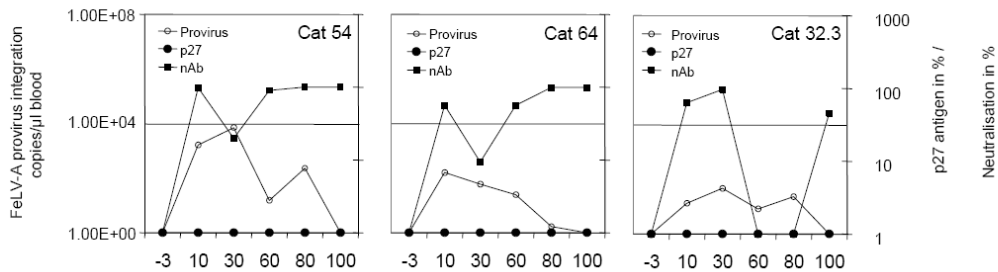
p15E and Leucogen vaccinated cats
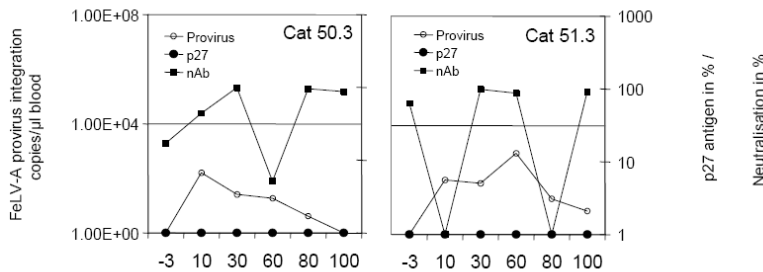
Figure 30 Kinetics of provirus integration in copies/µl blood, prevalence of p27 antigen in percentage and neutralising activity (measured at a serum dilution of 1:100 in % relative to preimmune serum) in cats.

3.6 Induction of neutralising antibodies against HIV-1 gp41

In order to transfer the model of the induction of neutralising antibodies against FeLV-A p15E to the HIV-1 transmembrane envelope protein gp41, recombinant hybrid proteins were designed. The hybrid proteins consisted of the p15E backbone and a gp41 sequence containing the 2F5 and the 4E10 epitopes. Using p15E of FeLV as backbone, we wished to determine whether substitution of the E2 domain of p15E by the E2 (2F5/4E10) domain of HIV-1 allows the induction of neutralising antibodies specific for HIV-1. The same modifications were performed as described for the FeLV-A ΔISU p15E protein (see 3.3.2) removing the isu domain and the opposing amino acid sequence at the C-terminal part including sequences for peptides J and K (Nick et al., 1990) (Fig. 31).

3.6.1 Characterisation of the antigen

Two hybrid proteins were generated differing in size of 9 amino acids in their C-terminal gp41 sequence. The p15E/gp41 hybrid I has a size of 9.9kDa and the p15E/gp41 hybrid II has a size of 8.9kDa plus the additional 4kDa of the calmodulin binding peptide (CBP) at their N-terminus for both proteins. The p15E/gp41 hybrid proteins I and II and were recognised by the mAb2F5 in Western blot analysis (Fig.34B) confirming the presence of the corresponding epitope in the proteins. In addition the hybrid I was detected by a minimum concentration of 4ng/ml mAb2F5 in ELISA (Fig. 32C). 10 rats (groups 71, 79 and 91) were immunised with p15E/gp41 hybrid protein I and 4 rats (group 90) were immunised with hybrid protein II. Additionally 3 rats (group 80) were immunised with p15E/gp41 hybrid I as described and boostered with 0.1mg gp41-derived peptide once.
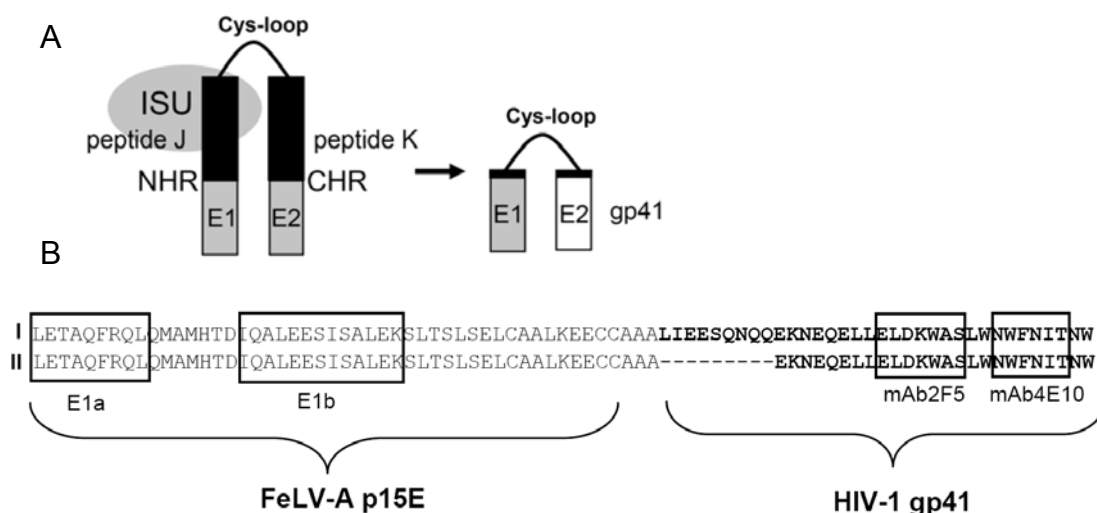
Figure 31 Properties of the p15E/gp41 hybrid proteins I and II. (**A**) Schematic presentation of the p15E ectodomain of FeLV and the modified p15E/gp41 hybrid proteins I and II. The localisation of the isu domain, the peptides J and K, the cystein-cystein loop as well as the N- and C-helix regions (NHR, CHR) and the epitope regions (E1, E2) are indicated. The white epitope region indicates the C-terminal gp41 sequence. (**B**) Amino acid sequence of the p15E/gp41 hybrid proteins. The p15E/gp41 hybrid I contains 9 additional amino acids of gp41 than the p15E/gp41 hybrid II. The C-terminal gp41 sequence is printed in bold. Epitopes in FeLV-A p15E (E1a, E1b) and in HIV-1 gp41 (mAb2F5, mAb4E10) are indicated.
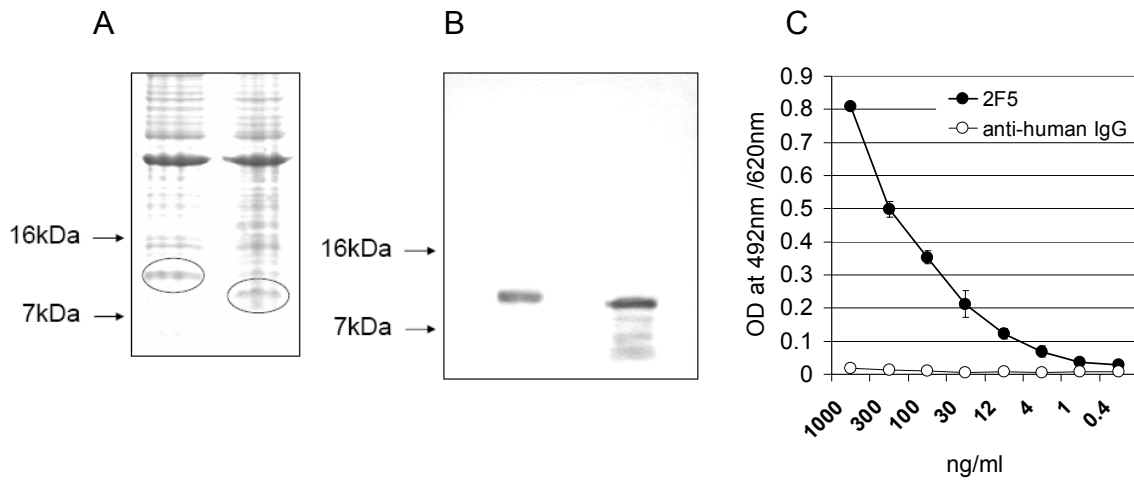
Figure 32 Characterisation of the p15E/gp41 hybrid proteins. (**A**)SDS-Page of p15E/gp41 hybrid I (1) and II (2) indicated in red circles and (**B**) detection of the p15E/gp41 hybrid I (1) and II (2) in a Western blot analysis with mAb 2F5 (0.5mg/ml). 1µg of hybrid protein suspension was loaded per lane. (**D**) Detection of the p15E/gp41 hybrid I by serial dilutions of mAb2F5 in ELISA. The secondary anti-human IgG was used as negative control.

3.6.2 Characterisation of neutralising antibodies

The neutralising efficacy of the sera was analysed in neutralisation assays using HIV-1IIIB in C8166 cells as well as the primary isolates HIV-1Bal and SF162 in freshly isolated human macrophages. Neutralisation was defined as a reduction of provirus integration for a minimum of 50%. Of 10 rats immunised and boostered with the p15E/gp41 hybrid I, 5 rats showed a neutralisation of up to 99% of the HIV-1IIIB (Fig. 33A; Tab.6) with titres of neutralising antibodies up to 1:16 (Fig. 33B; Tab.6). Using the primary isolates HIV-1Bal and HIV-1SF162 and human macrophages, two antisera (rats 71.2, 79.3) neutralised up to 81% at serum dilutions of 1:8 (Fig. 33C, D). In order to minimize the possibility of a cytotoxic effect obtained here, a synchronous neutralisation assay with concentrated antiserum from rat 79.4 and PERV was performed on C8166 cells. It was shown that antisera neutralising HIV-1 had not such an effect on the infection of C8166 cells by PERV (Fig. 33E).
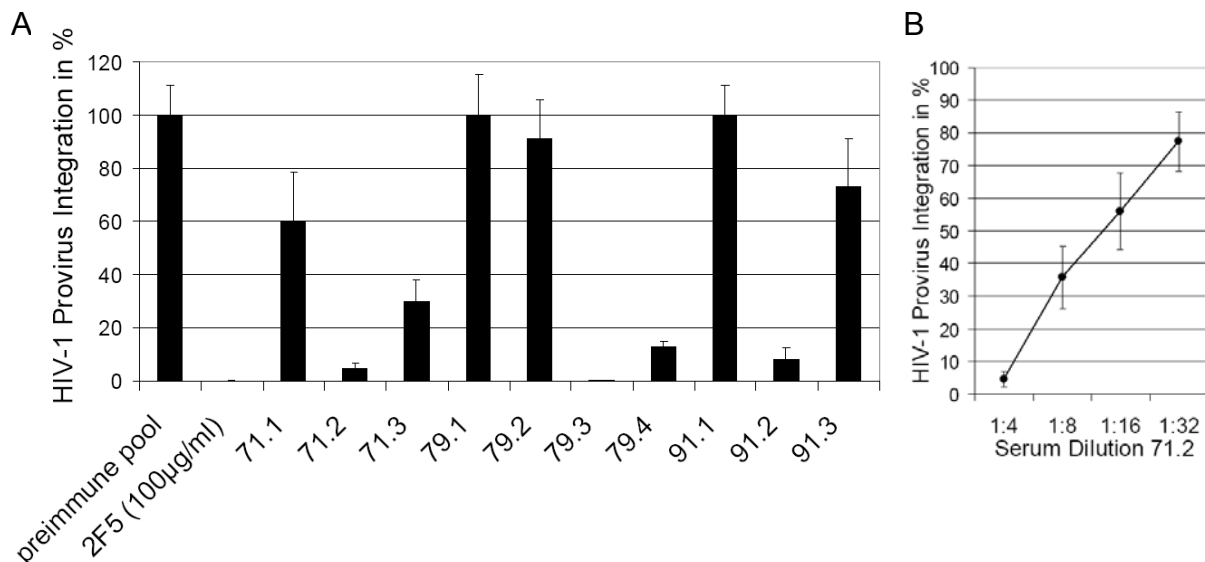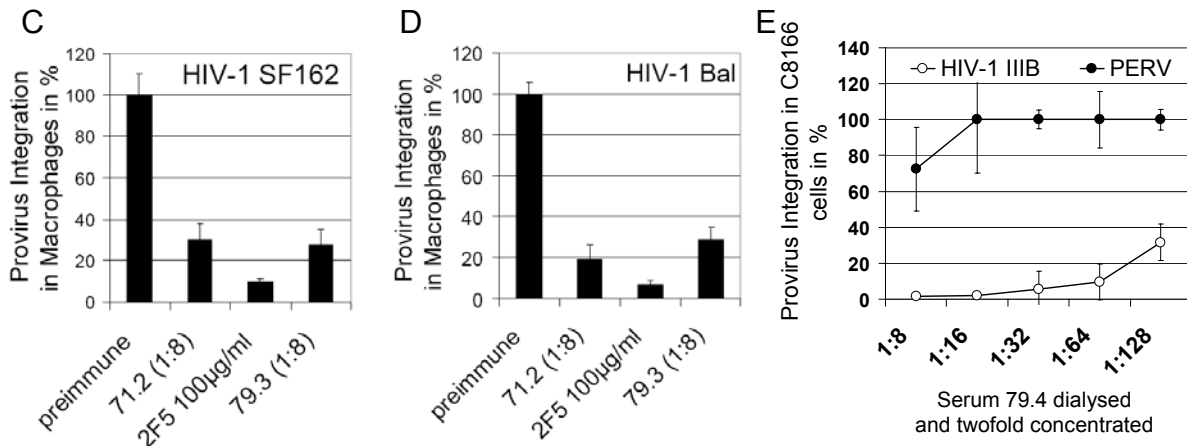
Figure 33 Neutralisation of HIV-1IIIB and of two HIV-1 primary isolates by rat antisera induced by immunisation with the p15E/gp41 hybrid protein I. Virus infection was measured as provirus integration by quantitative real time PCR. The mAb2F5 was used as positive control at a concentration of 100µg/ml. (**A**) HIV-1IIIB provirus integration in C8166 cells in percentage. Treatment with the preimmune serum was taken as 100%, all sera were used at a dilution of 1:4. (**B**) Inhibition of integration of HIV-1IIIB by rat serum 71.2 is dosis-dependend as shown by serial dilution of the serum. (**C, D**) Inhibition of provirus integration of HIV-1 SF162 (**C**) and of HIV-1 Bal (**D**) in donor macrophages after treatment with rat immune sera 71.2 and 79.3, both sera were used at dilution 1:8. Preimmune serum and mAb2F5 were used as controls. (**E**) HIV-1 IIIB and PERV provirus integration in C8166 cells in percentage treated with dialysed and twofold concentrated serum from rat 79.4.

Antisera obtained from animals immunised and boostered with p15E/gp41 hybrid II (Fig. 34; Tab.6) or immunised with p15E/gp41 hybrid I and boostered with the gp41-derived peptide E2 did not significantly neutralised HIV-1IIIB.

Analysing the rat sera in an ELISA using a gp41-derived peptide E2 containing the 2F5/4E10 epitope, titres of binding antibodies ranging between 1:400 and 1:6400 were found (Tab.6), indicating the induction of gp41-specific antibodies. However, no correlation between binding antibody titres and neutralisation titres was observed.
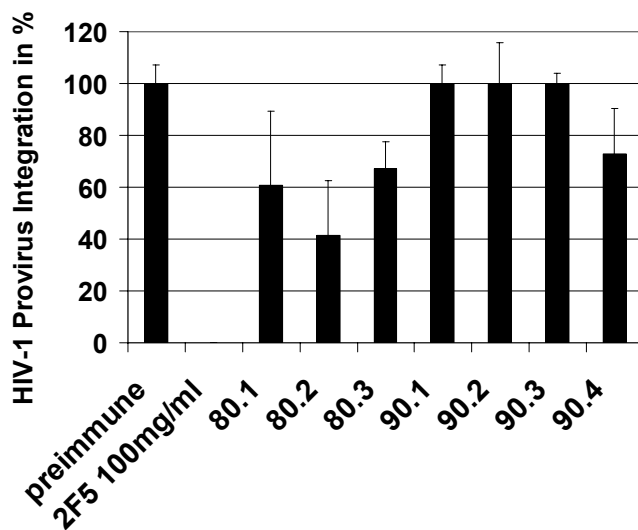
Figure 34 Neutralisation efficacy of HIV-1 IIIB by rat antisera induced by immunisation with p15E/gp41 hybrid I and boostered with gp41-derived peptide E2 (group 80) or by immunisation and boost with the p15E/gp41 hybrid II (group 90). Virus infection was measured as provirus integration by quantitative real time PCR in C8166 cells in percentage. The mAb 2F5 was used as positive control at a concentration of 100µg/ml. Treatment with the preimmune serum was taken as 100%, all sera were used at a dilution of 1:4.

3.6.1 Epitope mapping

In order to identify the epitopes recognised by the neutralising antisera, an epitope mapping was performed using pepspot membranes with overlapping peptides corresponding to the entire gp41 sequence of HIV-1 and to the ectodomain of p15E of FeLV-A (Fig. 37A, B). Epitopes were identified for gp41 located C-terminal close to the 2F5 epitope ELDKWA (QQEKNEQELL, EKNEQELLE) or in one case (rat 71.2) partially overlapping with the 2F5 epitope (QELL**ELDKW**) and in another case (rat 91.2) overlapping both, 2F5 and 4E10 (DKWASLWNWFNI) (Fig. 35C; Tab.6). In addition, one epitope was also detected on the p15E backbone (aa494-500: ALEESIS) (Fig. 35B; Tab.6), corresponding to the epitope described for antisera induced by immunisation of rats with p15E of FeLV and designated E1b (chapter 3.1).
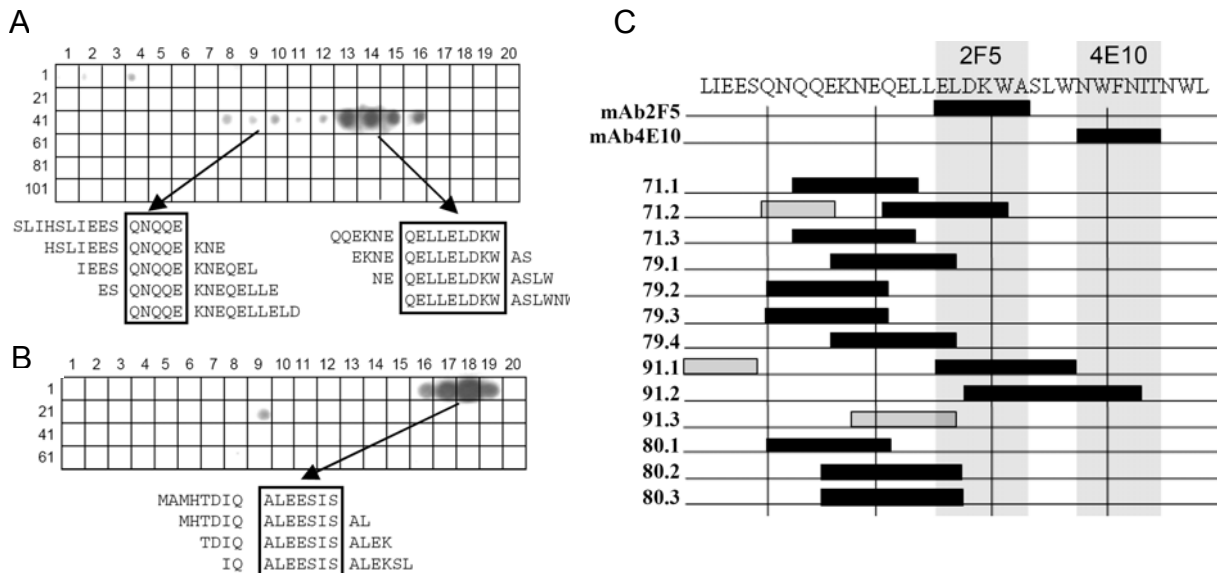
Figure 35 Epitope mapping using pepspot membranes with overlapping peptides and rat sera after immunisation with the p15E/gp41 hybrid proteins. The ECL method was used for detection. (**A, B**) Results obtained with the neutralising rat serum 71.2 using overlapping peptides corresponding to the entire gp41 of HIV-1 (**A**) and overlapping peptides corresponding to the entire p15E of FeLV (**B**). The sequences of the reacting peptides are shown and the identified epitopes are framed. (**C**) Summary of the epitope mapping of all rat antisera using gp41-derived peptides compared with the mAb2F5 and mAb4E10. Epitopes were identified as shown in A and B.

Table 6 Summary of immunisation studies with p15E/gp41 hybrid proteins I and II

Rat	Prime, Boost[b]	Elisa Titre[c]	Neutralisation titre in RTQ	Neutralisation in RTQ[d]	Epitope on C-terminal gp41
79.1	0.1mg p15E/gp41 hybrid I	6.4×10^3	0	0%	EKNEQELL**E**
79.2		6.4×10^3	0	0%	QNQQEKNE
79.3		6.4×10^3	**1:16**	**99%**	QNQQEKNE
79.4[a]		1.6×10^3	**1:16**	**85%**	EKNEQELL**E**
71.1		4×10^2	0	0%	QQEKNEQE
71.2		6.4×10^3	**1:16**	**95%**	QELL**ELDKW**
71.3		1.6×10^3	**1:8**	**70%**	QQEKNEQE
91.1		1.6×10^3	0	0%	**ELDKWA**SLW
91.2		1.6×10^3	**≥ 1:4**	**90%**	**DKWA**SLWNFN
91.3		1.6×10^3	0	0%	NEQELL**EL**
80.1	0.1mg p15E/gp41 hybrid I, E2-peptide	1.6×10^3	0	0%	QNQQEKNE
80.2		1.6×10^3	0	0%	EKNEQELL**E**
80.3		1.6×10^3	0	0%	EKNEQELL**E**
80.4		1.6×10^3	0	0%	n.d
90.1	0.1mg p15E/gp41 hybrid II	n.d	0	0%	n.d
90.2		n.d	0	0%	n.d
90.3		n.d	0	0%	n.d
90.4		n.d	0	0%	n.d

[a] generation of hybdridoma cells
[b] Groups 79, 71, 80 and 90 immunised with Montanide ISA 720, Group 91 immunised with Freund´s adjuvant
[c] Peptide-ELISA on HIV-1 E2 peptide: EKNEQELLELDKWASLWNWFNITNWL
[d] Neutralisation in percentage obtained at a serum dilution of 1:4

3.7 Generation of hybridoma cultures against p15E/gp41 hybrid protein I

By the generation of monoclonal antibodies from the spleen of rat 79.4 (chapter 3.5) previously shown to have an HIV-1 neutralising serum after immunisation with the p15E/gp41 hybrid I protein, it should be analysed if a single antibody population is responsible for the neutralising effect. Therefore spleen cells from rat 79.4 were fused to mouse myeloma cells and screened for gp41 specific monoclonal antibodies.

3.7.1 Characterisation of monoclonal antibodies binding to the HIV-1 gp41 E2 peptide

21 days after the fusion of spleen cells from rat 79.4 with mouse myeloma cells P3-X63-Ag8.653 most of the hybridoma cell cultures were grown confluent (Fig.36) and screening of hybridoma supernatants on HIV-1 E2 peptide was performed.
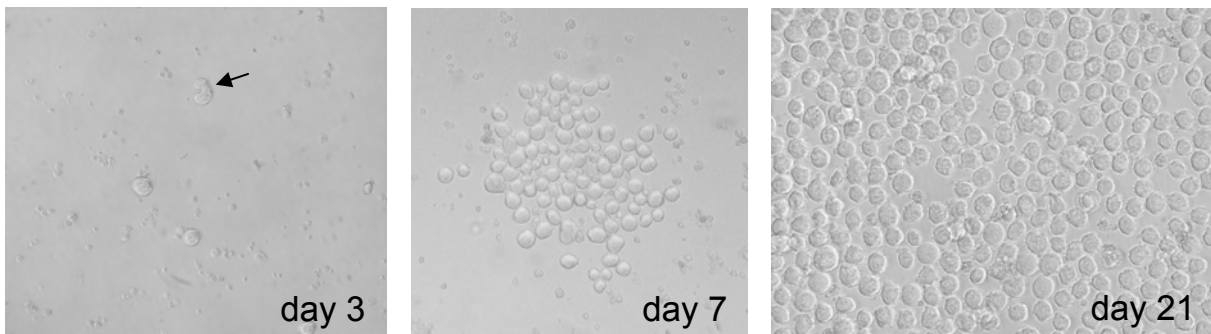
Figure 36 Hybridoma cell cultures at days 3, 7 and 21 after fusion at a magnification of 200x.

Out of 576 hybridoma supernatants tested 14 were shown to react with the E2 peptide and were subsequently singularised twice during hybridoma cell culture passages accompanied by continuous ELISA screening (Fig. 37).
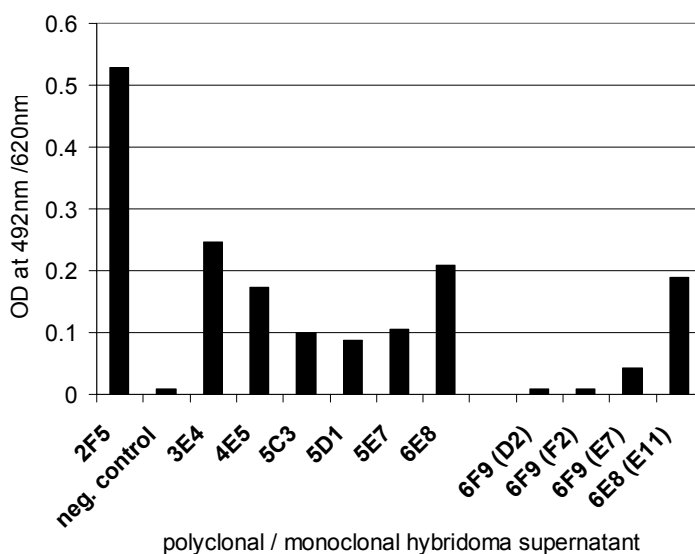
Figure 37 Example of an ELISA screening for hybridoma cell culture supernatants after first and second singling of clones. Clones with denotations in brackets were singularised twice and supposed to be monoclonal.

Two monoclonal antibodies could be identified binding specifically to the sequence of HIV-1 gp41 in epitope mapping (mAb3E4E3 and mAb6E8E11) (Fig 38). However, the mAb6E8E11 binds to a sequence which has not been included in the sequence of the p15E/gp41 hybrid protein I (WMEWDR) (Fig. 38A, C). The mAb3E4E3 recognises three sequences on the HIV-1 gp41 peptide membrane also including the sequence WMEWDR. In addition it detects the sequences SLIHSL and QELLELDKW (Fig. 38B). The first one is located N-terminal shortly before the gp41 sequence included into the p15E/gp41 hybrid used for immunisation and the second epitope is in part identical to the epitope detected by rat serum 79.4 (EKNE**QELLE**) (chapter 3.6, Fig. 35C).
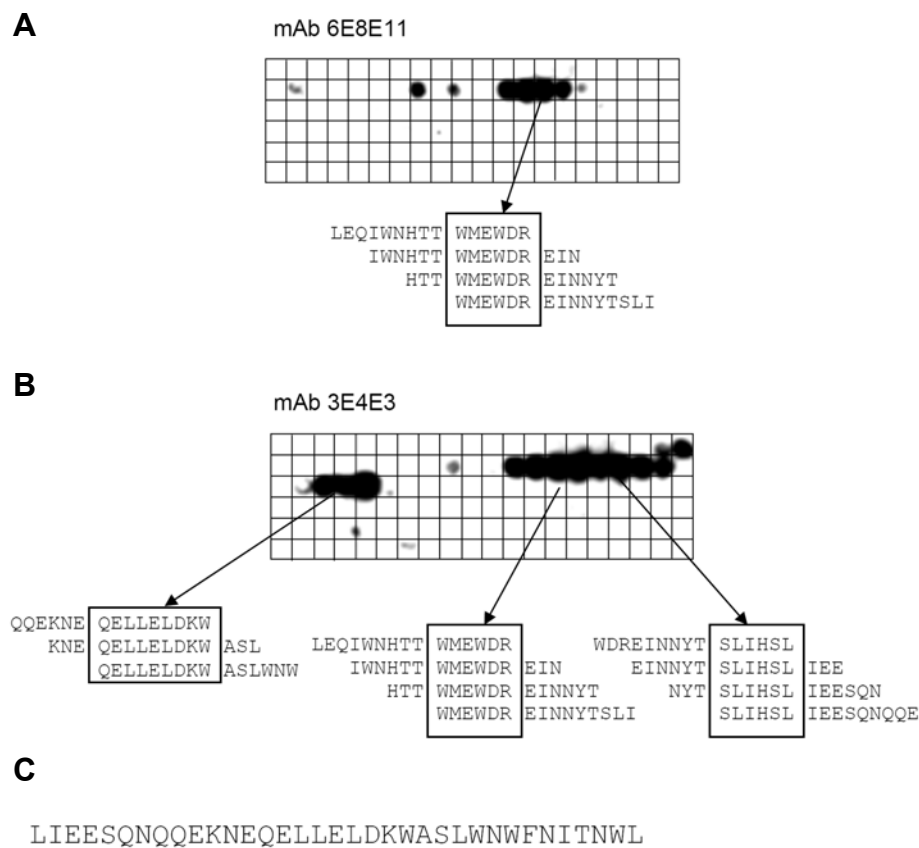
Figure 38 Epitope mapping using pepspot membranes with overlapping peptides and hybridoma cell culture supernatants. (**A**) Results obtained with the mAb 6E8E11 and (**B**) results obtained with the mAb 3E4E3. The sequences of the reacting peptides are shown and the identified epitopes are framed. (**C**) C-terminal HIV-gp41 sequence included in the p15E/gp41 hybrid protein I used for immunisation of rat 79.4

The mAb´s 3E4E3 and 6E8E11 were shown to bind specifically to the gp41 E2 peptide as well as to the recombinant p15E/gp41 hybrid protein. For the cell culture supernatant of the mAb3E4E3 an ELISA titre of $\geq$ 1:2187 (Fig. 39A) and for the mAb6E8E11 an ELISA titre of 1:256 (Fig. 39B) on the gp41 E2 was observed. However, none of them showed a neutralising activity in an HIV-1 neutralisation assay.
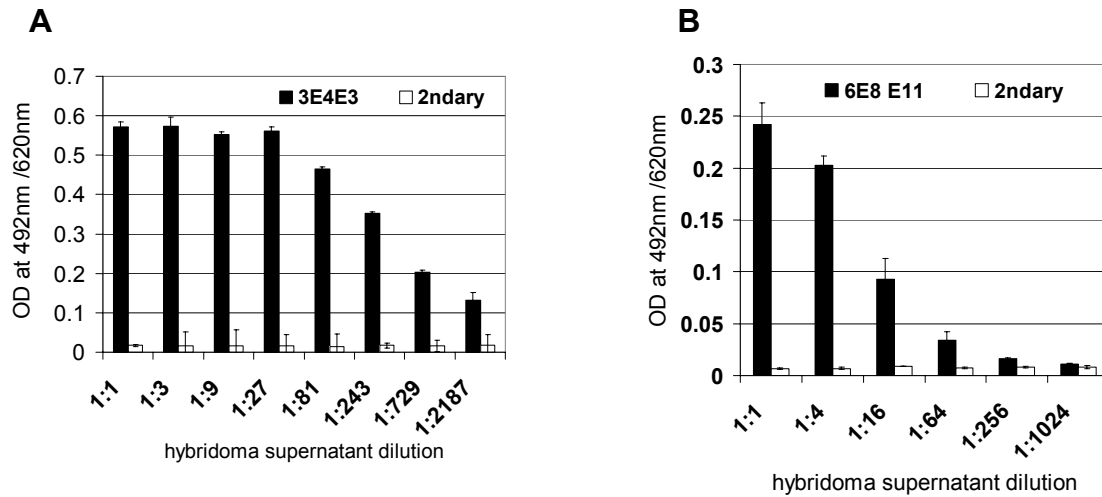
Figure 39 Reactivity of mAb3E4E3 in a three fold serial dilution **(A)** and of mAb6E8E11 in a four fold serial dilution **(B)** with the HIV-1 gp41 E2 peptide.

4. Discussion

4.1 Induction and characterisation of neutralising antibodies against FeLV-A p15E in different species

Vaccines against FeLV represent the first example of successful vaccines preventing a retroviral disease. Three types of FeLV vaccines are currently available: inactivated whole virus preparations, inactivated mixed subunit preparations from FeLV-infected tissue culture filtrate and recombinant FeLV proteins. The commercially available vaccines containing inactivated FeLV subunit preparations are Fevaxyn, Leucocine and Leukocell2. Leucogen is an example of a recombinant vaccine, and comprises recombinant non-glycosylated surface envelope protein p45 (Tab.1).

None of the vaccines regularly induce virus neutralising antibodies following vaccination: such antibodies are usually detected only after recovery from challenge (Jarrett, 2001). However, a high proportion of cats are protected by vaccination, indicating that immune mechanisms other than neutralising antibodies may be involved. This conclusion is confirmed by results showing that a DNA vaccine engineered to promote the induction of FeLV-specific cytotoxic T cells provided protection against FeLV challenge without any detectable antibody responses (Hanlon et al., 2001). However, it should not be concluded from these findings, that neutralising antibodies are not important. The presence of neutralising antibodies in cats recovering from natural FeLV infection clearly correlates with resistance to infection and passive transfer of antibodies, either naturally through the colostrum (Hoover *et al.,* 1977) or experimentally by infusion (Haley et al., 1985) protects cats against FeLV challenge. It is still possible that the methods used to detect neutralising antibodies are ineffective and that the level of such antibodies required to protect are lower than the detection threshold. Data presented here clearly show that the immunisation with Leucogen alone (group 56) was able to induce neutralising antibodies (group 56, Fig. 19, 20; Tab.4).

Numerous attempts have been undertaken to improve the efficacy of vaccination. For example, immunisation with the immunostimulating complexes (ISCOM) containing the gp70/gp85 precursor molecules not only induced virus neutralising antibodies, but also protection against infection (Osterhaus *et al.,* 1985) . The development of antibodies to gp70 and p15E were confirmed by Western blot (Osterhaus *et al.,* 1989). When compared with the commercial vaccine Leucocell, containing inactivated FeLV, the ISCOM preparation proved to be superior. Virus neutralising antibodies were also induced by synthetic peptides corresponding to a domain of gp70 of FeLV-A involved in infection (Weijer *et al.,* 1993). Since FeLV-B and FeLV-C might originate by recombination between FeLV and endogenous FeLV-related sequences (Bechtel *et al.,* 1998), neutralising antibodies against FeLV-A should also protect cats from natural infection with all subgroups.

Some commercial vaccines such as Leucogen, which comprise the 45 kDa unglycosylated surface envelope protein, do not contain p15E and for others containing p15E the role of this protein in the

induction of neutralising antibodies has not been addressed. For the first generation vaccines that do not fully protect animals from infection, addition of p15E protein as an additional component may be of great benefit.

4.1.1 Immunisation with FeLV-A p15E induces neutralising antibodies in different species including cats

As it had been shown for murine leukaemia virus (MuLV) that immunisation with gp70 plus p15E was more effective in inducing neutralising antibodies than was immunisation with gp70 alone (Schwarz et al., 1984), the ability of FeLV-A p15E to induce neutralising antibodies was studied in a goat, in rats and finally in cats. By immunisations of a goat and of rats with the ectodomain of FeLV-A p15E binding antibody titres up to 1×10^6 were induced and neutralisation was observed from 51% to 99% (chapter 3.1, Tab.3). For antisera induced by p15E immunisations in rats neutralisation titres up to 1:16 were found (chapter 3.3, Fig. 19, C, F, Tab. 4), equivalent to an amount of 150-300µg/ml of p15E specific antibodies (chapter 3.1, Fig. 11). This observance is in accordance with previously published data showing the induction of neutralising antibodies by immunisation with the p15E protein of the gammaretrovirus PERV (Fiebig et al., 2003). Moreover it was shown that the efficiency of neutralising antibodies induced by immunisation with FeLV-A p15E is more than 50 fold increased in its natural host. Compared to the goat and rats, cats showed neutralisation titres up to 1:1024 (chapter 3.4 Fig. 24, Tab. 5) although titres of binding antibodies were similar to those observed in a goat and in rats (chapter 3.4, Tab.5).

4.1.2 The humoral immune response against the transmembrane proteins of HIV, PERV and FeLV-A shows the detection of similar located epitopes

For antisera obtained from immunisation with p15E in a goat, in rats and in cats two linear epitope regions were identified in the ectodomain of FeLV-A p15E termed E1 and E2 each subdivided into two epitopes E1a, b and E2 a, b (Fig. 42 C). In a previous study, several regions of gp70 and of p15E able to elicit FeLV-neutralising antibodies were identified by immunisation with synthetic peptides coupled to a carrier protein (Elder et al., 1987). In this case, the peptides corresponded to the Gardner-Arnstein virus and the antisera were induced in rabbits. Interestingly, one of the peptides inducing neutralising antibodies, I-7 (corresponding to the C-terminal end of p15E) contained the epitope E2b that was detected here and after immunisation with PERV p15E (Fiebig et al., 2003). In addition, the peptides C-18 and I-6 also induced neutralising antibodies and contained the epitopes E1b and E2a, respectively (Elder et al., 1987). However, no detailed epitope mapping of the neutralising rabbit sera had been performed.

Of greatest interest is the similar location and partial sequence homology of the epitopes described for

p15E of PERV (Fiebig et al., 2003) and FeLV (Fig. 25) with epitopes described for HIV. Monoclonal antibodies that neutralise a broad spectrum of HIV subtypes have been generated using antibody-producing cells from HIV-infected patients. The first such antibody, 2F5 (Muster et al., 1993), recognises a linear epitope ELDKWA localised at the N-terminal end of gp41 and the second, 4E10, recognises the epitope NWFNIT (chapter 3.3, Fig. 25). This epitope has partial homology to the E2 epitope of PERV (FEGWFN) (Fiebig et al., 2003) and the E2b epitope described here for FeLV (WFEGWFN). Although this epitope was not recognised by the FeLV p15E-specific goat serum, it was detected as the major C-terminal epitope by the sera from the immunised rats. In gp41, the 4E10 and the 2F5 epitopes are located in close proximity (LL**ELDKWA**SLWN**NWFNIT**) (Fig. 40A). A second epitope, N-terminal to FEGWFN, was also detected by the FeLV p15E-specific goat and rat sera (Fig. 40C). This second epitope, designated E2a, contained (in the case of the goat serum) the core sequence KQRQQLF. Although the distance between these two epitopes (RL**KQRQQLF**DSQQGW**FEGWFN**K) is much greater than between the 2F5 and the 4E10 epitopes, their general proximity and immunogenicity suggests that the E2a and E2b epitopes may represent the functional equivalents of the 2F5 and 4E10 epitopes, respectively.

It is noteworthy that the recognition of the epitopes is dependent on the different species. The neutralising goat serum specific for PERV p15E recognised the 4E10 equivalent in the C-terminal end and the epitope E1a in the N-terminal end (Fiebig et al., 2003) (Fig.40B). The FeLV p15E specific goat serum recognised the 2F5 equivalent in the C-terminal end and E1a in the N-terminal end (Fig. 25, 40C). In contrast, sera from rats immunised with PERV p15E recognised a 4E10 equivalent and/or a 2F5 equivalent in the C-terminal end and an E1a epitope in the N-terminal end (Fiebig et al., in preparation) (Fig. 40B). Sera from rats immunised with FeLV p15E recognised a 4E10 equivalent and/or a 2F5 equivalent in the C-terminal end and E1a and/or E1b in the N-terminal end (Fig. 40C).

Immunisation of cats with p15E of FeLV-A induced neutralising antibodies, which recognised epitopes similar to those described for sera after immunisation of rats and goat with FeLV-A and PERV p15E (Fig. 25). Interestingly, cats infected with FeLV also develop antibodies against p15E, although the reactions by immunoblot are weak and ELISA titres are low (Tab.5, Fig.23). Epitope mapping revealed a variety of epitopes recognised by sera from FeLV-infected animals, including epitopes detected by sera from p15E-immunised cats, albeit comparatively weakly (chapter 3.3, Fig. 25). This suggests that natural FeLV infection results in a weak induction of antibodies specific for the viral transmembrane protein p15E and a low induction of neutralising antibodies.
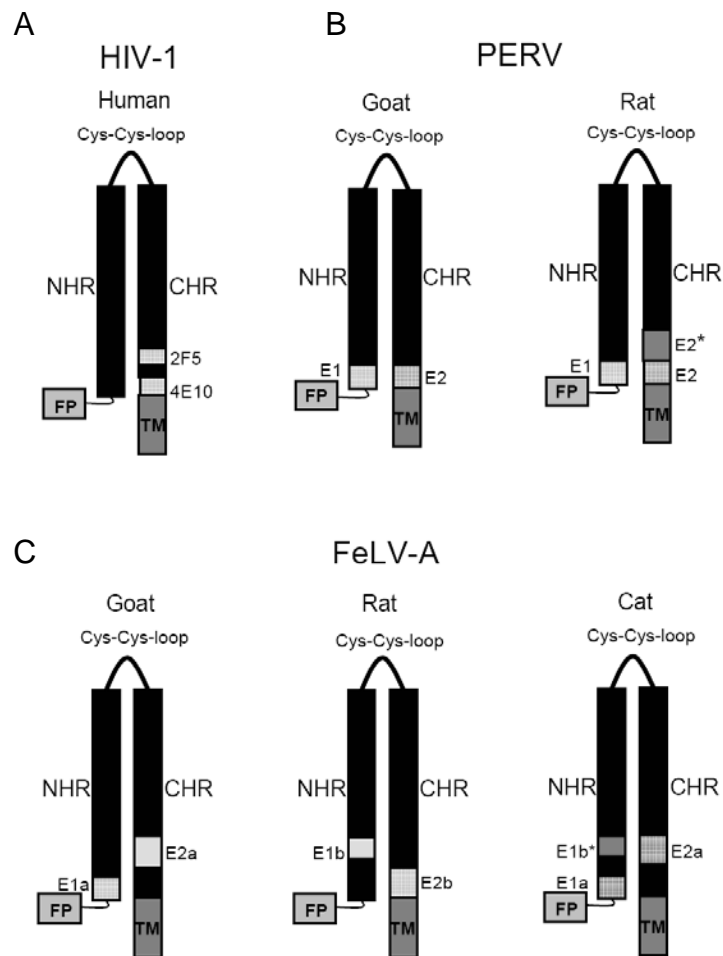
Figure 40 Schematic presentation of epitopes recognized in the ectodomains of transmembrane proteins of HIV-1, PERV and FeLV-A. Indicated are the cystein-loop (Cys-Cys-loop), the N- and C-terminal helix regions (NHR, CHR), the fusion peptide (FP) and the transmembrane domain (TM). (**A**) Localization of the 2F5 and the 4E10 epitopes in HIV gp41. (**B**) Detection of epitopes E1 and E2 induced by immunisation of PERV p15E in a goat and in rats. The E2* epitope is sporadically detected in rats. (**C**) Detection of epitopes E1a, b and E2a, b induced by immunisation of FeLV-A p15E in a goat, in rats and in cats. The E1b epitope is sporadically detected in immunised cats and epitopes E1a, b and E2a also could be detected in FeLV-A infected cats.

Synthetic peptides corresponding to the PERV E1 and E2 epitopes inhibit the neutralising activity of the goat serum more effectively when added together and it was therefore proposed they represent a conformational epitope (Fiebig et al., 2003). Although there is still no clear evidence for this, recent studies have shown that conformational changes take place in the transmembrane envelope protein of all retroviruses during the infection process that bring the two epitopes into close proximity (Gallo et al., 2003). After binding of the surface envelope protein (gp70 for FeLV and PERV and gp120 for HIV-1) to their corresponding receptors, the N-terminal helix and the C-terminal helix interact (E1 and

E2 are located at the N-terminal and C-terminal ends, respectively). The neutralising antibodies may inhibit either the interaction of both helices or later events in the infection process.

The mechanism of action of the human mAb2F5 is still poorly understood, although it is known that the antibody binds to the virion before attachment of the virus to the cell (Sattentau et al., 1993; Schmolke et al., in preparation). It was also shown that 2F5 does not influence the interaction between the helices (Golding et al., 2002) but rather inhibited later steps during infection (de Rosny et al., 2004). All attempts to induce neutralising antibodies using recombinant proteins containing the E2 epitope of HIV-1 gp41 (ELDKWA), have so far failed (Tian et al., 2001; Liao et al, 2000; Lu et al., 2000). Although final proof has to be presented, e.g., by immunoprecipitation of purified virus, binding of the HIV neutralising antibody 2F5 to the virions before attachment to the cells was shown (Sattentau et al., 1993). Also p15-specific antibodies induced in rats (Fig. 14) and cats (Fig. 26) were able to bind to the cell surface of infected cells as shown in immunofluorescence studies, suggesting the availability of the epitopes on the virion itself despite the fact that most of the transmembrane envelope protein is occluded by the surface envelope protein gp70.

These data obtained from the epitope characterisation show a greater similarity than previously suspected between gammaretroviruses such as PERV and FeLV and lentiviruses such as HIV-1 with regard to the localisation of target epitopes for broadly neutralising antibodies in the transmembrane envelope proteins. This similarity may be based on a common requirement for conformational changes during retroviral infection that can be inhibited by antibodies and hence prevent later stages of virus internalisation.

4.1.3 The deletion of the ISU domain from FeLV-A p15E does not improve the antigen, but maintains an identical humoral immune response as observed for immunisation with p15E

Due to the immunosuppressive properties of the transmembrane envelope protein, many researchers and manufacturers argue that p15E should not be added to a FeLV vaccine. Indeed, it has been shown that p15E and a synthetic peptide corresponding to a highly conserved region in the transmembrane envelope protein of all retroviruses (immunosuppressive domain ISU) inhibit mitogen-triggered lymphocyte activation and modulate cytokine production (Hebebrand et al., 1979; Mathes et al., 1979; Denner et al., 1998, Cianciolo et al., 1985). To evaluate the influence of ISU on the immune response, three rats were immunised with a p15E derived recombinant protein lacking the N-terminal located immunosuppressive domain and a C-terminal opposing amino acid sequence (FeLV-A ΔISU p15E). However, no difference was observed in the characterisation of the humoral immune response in comparison to immunisations with the complete p15E ectodomain. The induction of binding antibody

titres ranged from 2.5×10^5 and 1×10^6 (Fig. 16A) being similar to those induced by the complete p15E ectodomain. Epitopes in the E1 as well as in the E2 region were detected (Fig. 17) and one serum showed neutralisation of FeLV-A with a titre up to 1:16 (Fig. 16B).

Interestingly the neutralising antiserum (70.1) not only showed the highest titre of binding antibodies but also showed the recognition of the N-terminal E1a and E1b epitopes plus the C-terminal E2a epitope (Fig. 17). The other two antisera (70.2 and 70.3) only detected epitopes within the E1 region and had not such a neutralising activity. Thus it can be assumed that (i) the immunosuppressive domain does not have a major influence on the induction of the humoral immune response against FeLV-A p15E and (ii) that an induction of antibodies specific for epitope regions E1 and additional specific for the epitope region E2 might be necessary for an effective neutralisation of FeLV-A.

4.1.4 Sequences homologue to the epitopes are present as endogenous retroviral sequences in cats

Comparing the sequence of the infectious FeLV-A, able to induce leukemia and immunodeficiency in infected cats, with that of an endogenous proviruses, revealed a strong homology in the epitopes E1a, E1b, E2a and E2b. Only the E2b sequence (DGL instead of GWF) and regions outside of these the epitopes showed differences (Fig. 25). The induction of binding and neutralising antibodies specific for sequences present as endogenous retroviruses in the genome of all cats indicates a lack of tolerance and suggests that expression of the endogenous viral genes during ontogenesis (when discrimination between self and non-self is made) does not occur. Similar observations have been made with human endogenous retroviruses (HERVs), especially HERV-K, which is expressed in human teratocarcinomas (Löwer et al., 1996) and melanomas (Büscher et al., 2005, Muster et al., 2003). Antibodies against HERV-K were found in 45% (45 of 100) of testicular tumour patients, 26% (31 of 120) of lymphoma patients and 38% (3 of 8) of multiparous pregnant women (Löwer et al., 1996). Furthermore, antibodies against the transmembrane envelope protein were found in 22% (13 of 60) of melanoma patients (Muster et al., 2003). Although antibody titres are elevated compared with normal blood donors (3%, 1 of 30), they hardly ever reach the titres seen after infection with exogenous retroviruses such as HIV. Nevertheless, it is intriguing that antibodies are produced at all, since HERV proteins (like the proteins of endogenous retroviruses in cats) can be regarded as self-antigens that should induce tolerance. However, the presence of antibodies suggests that tolerance is not induced and that induction of antibodies specific for endogenous retroviral proteins is possible both by immunisation and by infection with an exogenous but highly related leukaemia virus. Absence of tolerance to retroviral proteins is certainly characteristic for all species carrying endogenous retroviruses.

4.1.5 Combined immunisation with FeLV-A p15E and Leucogen induces an increased response of neutralising antibodies in rats

Immunotherapy studies in AKR mice that spontaneously develop leukaemia showed that although neither antibodies to gp70 nor antibodies to p15E could influence the course of leukaemia development, a combination of the two antibodies was effective (Thiel et al., 1987). In parallel it has been shown for exogenous murine leukaemia virus (MuLV) that immunisation with gp70 and p15E was more effective in inducing neutralising antibodies than was immunisation with gp70 alone (Kleiser et al. 1986), and we therefore studied the ability of FeLV p15E to improve vaccination with Leucogen containing the unglycosylated form of gp70.

Results from immunisation of rats with FeLV-A p15E were confirmed here, showing that with and without simultaneous immunisation with Leucogen epitopes E1a, b and E2a, b were detected (Fig.22). There are three important conclusions from these results. First, sera induced with 100μg or 500μg p15E (groups 55 and 57) always detected the E2b epitope, while the E1b epitope was recognised only by one antiserum of each group (55.2 and 57.1). However, only these two antisera efficiently neutralised FeLV (Fig. 21; Tab.4), indicating a critical role of the E1b epitope in virus neutralisation. Second, the E2 epitopes also seem to be crucial for neutralisation since the serum from animal 60.1 did not recognise E2 and had only a weak neutralisation activity when compared with the sera from two other animals of the same group that recognise E2 (Fig. 21). Third, as mentioned above, only two of the sera from animals immunised with p15E recognised E1b and only these sera were neutralising. On the other hand, sera from all animals immunised with p15E and Leucogen recognised E1b (Fig. 22; Tab.4), indicating that simultaneous immunisation of both antigens increased the recognition of the crucial epitope E1b.

When p15E and Leucogen were injected simultaneously at one site the titre of p45-specific binding antibodies was reduced significantly (Fig. 20; Tab.4). An antigenic dominance of the transmembrane may be a reason for this effect. In addition, p15E may interact with p45, hiding some, but not all epitopes. Since the neutralising activity was not impaired despite the lower titre of binding antibodies, epitopes involved in neutralisation were obviously not hidden.

The results clearly show that immunisation with both antigens induced higher titres of neutralising antibodies suggesting that combination of Leucogen and p15E may be the strategy of the future. In addition, simultaneous immunisation of both antigens increased also the recognition of an epitope in p15E crucial for neutralisation (E1b, Fig. 22; Tab.4). Further more by these findings assumptions from immunisation with FeLV-A ΔISU p15E were supported indicating that the recognition of an N-terminal (E1) and a C-terminal (E2) epitope is essential for an effective neutralisation of FeLV-A.

4.2 FeLV-A p15E vaccine studies in cats

4.2.1 Immunisation with FeLV-A p15E alone can protect cats from productive infection

Our previous findings that immunisation with the transmembrane protein of retroviruses, including HIV-1, PERV and FeLV, can induce virus neutralising antibodies, have here been extended to show that a vaccine comprising FeLV p15E protects a proportion of cats against challenge with live virus.

FeLV is an excellent system in which to develop vaccine strategies for retroviral infections. Although FeLV can establish a persistent viraemia in cats, which leads in most cases to the death of the animal within a few years, the majority of cats exposed to the virus recover naturally. Both virus neutralising antibodies and cytotoxic T cells (CTL) have been implicated in the immune response that leads to recovery (Flynn et al., 2002) and either antibodies (Hoover et al., 1977; Jarrett et al., 1977) or CTL (Flynn et al., 2000) on their own are known to protect cats from experimental infection. Cats that recover from natural infection appear to have a normal life expectancy (Hardy et al., 1984), despite the fact that many retain a latent virus infection in the bone marrow, and possibly other tissues, for many months or years (Pacitti & Jarrett, 1985), and proviral DNA is found in a sizeable proportion of apparently healthy, non-viraemic pet cats (Hofmann-Lehmann et al., 2001). The clinical significance of the retention of this covert infection is not known but the capacity to quantify proviral DNA may have prognostic value in future.

Provirus load is an important parameter characterising disease progression in other retroviral infections (Watson et al., 1997). In HIV and simian immunodeficiency virus (SIV) infection a primary peak of provirus load was described, which decreases with the onset of the virus-specific immune response (Stevenson, 2003). Later, in the asymptomatic phase, provirus load is relatively low, increasing again with progression to AIDS and reaching it highest value with full-blown AIDS. In the present experiment, in non-immunised cats infected with FeLV an acute viremia was observed with a maximum in proviral load at day 30 (Fig. 29). In the animals immunised with Leucogen, provirus load also increased up to day 30 but to a much lower level and then decreased, reaching zero in most cases at day 100. There was a good correlation between provirus load and p27 antigen load. Animals immunised with Leucogen or the combination of Leucogen and p15E had the lowest provirus load and were always p27 antigen negative. Non-immunised animals had the highest provirus load and the highest p27 antigen load. In a previous study, Hofmann-Lehmann et al. (2001) found that cats that had been experimentally infected with FeLV and were antigen negative had a low and decreasing provirus load and increasing antibody titress. These animals developed a regressive contained viremia. On the other hand, animals with high and increasing p27 antigenaemia had a high provirus load and low, if any, antibody titress. These animals developed a progressive persistent viremia. The non-immunised animals in our experiment are characterised by virus load and p27 antigenaemia consistent with a progressive viremia. By contrast, all animals immunised with Leucogen alone or in combination with

p15E and 3 of 6 animals immunised with p15E alone are characterised by a regressive, contained viremia (transiently antigenaemic or p27 antigen-negative).

All of the cats in the present experiment became provirus positive after FeLV challenge, indicating that neither the commercial vaccine Leucogen nor p15E protected from provirus acquisition and minimal viral replication. This result agrees with findings in another study with two other vaccines: Eurifel, a canarypox-vectored live vaccine containing FeLV-A *env, gag* and *pol,* and Fel-O-Vax, an inactivated whole virus FeLV vaccine, using similar methods (Hofmann-Lehmann et al., 2005). These vaccines also did not induce sterilising immunity. However, vaccinated cats that resist challenge develop a very powerful immune response and are likely to be solidly protected from further infection, and free from FeLV-related disease. Although half of the cats immunised with p15E were protected from a productive infection they showed a significantly higher provirus load in the peripheral blood than cats immunised with Leucogen or with Leucogen and p15E until the end of the study.

In all cats immunised with Leucogen, p15E or a combination of both, binding antibodies were found (Fig. 27). Interestingly, cats immunised with Eurifel (Hofmann-Lehmann et al., 2005) or experimental FeLV DNA vaccines (Hanlon et al; 2001; O'Donovan et al., 2005), which also conferred protection from viremia, did not develop antibodies at all. Antibodies developed only after challenge, indicating that the challenge virus had grown in the vaccinated cats. In contrast, animals immunised with Fel-O-Vax, had detectable FeLV specific antibodies prior to challenge and showed afterwards a strong anamnestic response (Hofmann-Lehmann et al., 2005). In the animals protected from antigenaemia after immunisation with Leucogen, p15E or a combination of both, the neutralising activity of their sera correlated with protection. The protected cats showed the highest levels of neutralisation activity four weeks after immunisation (except cat 51.3) and at day 100 after the challenge (Fig.27C, Fig. 28), while for the non-immunised control cats or the cats that were not protected by immunisation, significantly lower levels of neutralising antibodies were observed (Fig. 30). These data confirm previous findings showing that cats with contained viremia displayed a more pronounced humoral immune response (binding antibodies measured in an ELISA) than cats that became viraemic (Hofmann-Lehmann et al., 2001). Animals immunised with Leucogen alone did not have neutralising antibodies prior to virus exposure (Fig. 30). This result agrees with data obtained when cats were immunised with Eurifel or Fel-O-Vax (Hofmann-Lehmann et al., 2005). Most interestingly, 2 of 6 animals immunised with p15E alone (Fig. 30) and both animals immunised with the combination of Leucogen and p15E (Fig. 30) had neutralising antibodies before challenge. This is the first time that neutralising antibodies prior to challenge have been described. Cats immunised with Leucogen alone (Fig. 30) or with Eurifel or Fel-O-Vax (Hofmann-Lehmann et al., 2005) did not have detected neutralising antibodies prior to virus exposure. On the other hand, in the present experiment, neutralising antibodies appeared in all animals, including non-immunised animals after week 10.

Although there was no correlation between protection and the neutralising activity three days before the challenge (i.e. 4 months after the boost immunisation) (Fig. 28), there was a good correlation

between protection and the titres of neutralising antibodies in sera obtained immediately after the second, booster immunisation (except cat 51.3). This result suggests that the ability to induce protection and the efficacy of a vaccine might be tested immediately after immunisation by estimating the titre of neutralising antibodies at that time. Later the titre of neutralising antibodies decreased rapidly and could not be detected at challenge. Interestingly, the titre of neutralising antibodies induced by p15E declined less rapidly than those induced by Leucogen. This result may be due to the neutralising antibodies in each group of cats being directed at a different target in the virus: p15E in the case of the cats immunised with p15E and gp70 in the case of the cats immunised with Leucogen. In addition, it was clearly shown that the titre of neutralising antibodies, but not binding antibodies, is a critical parameter for the prediction of antibody mediated protective immunity.

In capter 3.3.2 in which the immunisation of rats with FeLV p15E or Leucogen or a combination of both is described, the combination induced significantly higher titres of neutralising antibodies compared to immunisation with single antigens (p15E or Leucogen alone). Consequently, it was anticipated that vaccination with the combination might improve the quality of the response to challenge. However, cats immunised with the combination did not have higher titres of neutralising antibodies than cats immunised with p15E alone (Fig. 27, Fig. 29).

These observations have essential implications for the development of antiretroviral vaccines including a vaccine against HIV-1. First, protection against challenge by vaccination against retroviruses using the transmembrane protein is possible in an outbred population. Secondly, the question of whether the induction of sterilising immunity against retroviruses is necessary to protect vaccinates from developing active persistent infection and disease is open. A HIV vaccine that did not induce sterilising immunity, but protected from viremia and disease would be a great success. However, cells with persistent integrated proviruses might finally start to produce virus, e.g. by immune activation, which might be followed by viremia, disease and virus transmission. Therefore a vaccine able to induce sterilising immunity would be of great advantage. Adoptive transfer studies using broadly neutralising antibodies directed against gp120 and gp41 of HIV- 1 (2F5 and 4E10) showed that sterilising immunity in a retroviral system is possible (Ruprecht et al., 2003). Better immunisation strategies, including combination of different viral targets and a better presentation of the transmembrane envelope protein may increase the neutralising activity of the induced antisera.

4.3 Induction of neutralising antibodies against HIV-1 gp41 in rats by immunisation with p15E/ gp41 hybrid protein I

According to the protein folding model shown in chapter 3.6, Fig.31A which is based on previous publications demonstrating interactions between the N-terminal helix and the C-terminal helix of retroviral transmembrane envelope proteins (Eckert and Kim 2001; Gallo et al., 2003) the E1 epitope in the N-terminal helix is located opposite to the 2F5/4E10 epitope domain in the C-terminal helix.

These findings support the data showing that antibodies neutralising PERV or FeLV induced by immunisation with their p15E recognised an N-terminal and a C-terminal epitope region exposed in close proximity after interaction of both helices during intramolecular conformational changes (Fiebig et al., 2003).

The data obtained here indicates the possibility of induction of antibodies neutralising HIV-1 of the type 2F5 and 4E10 using p15E of FeLV with the inserted E2 (2F5/4E10 epitope) domain of gp41 of HIV-1. This is the first report showing induction of 2F5/4E10-like neutralising antibodies despite numerous attempts by several other laboratories (Tian et al., 2001; Lu et al., 2000; McGaughy et al., 2004). In addition, a new epitope domain in gp41 recognised by the antisera induced by immunisation with the p15E/gp41 hybrid protein was described (QNQQEKNEQELLELDKW). The localisation near the 2F5 epitope suggests that epitopes within this domain are the target of the neutralising antibodies.. Recently binding of 2F5 as well as 4E10 to cardiolipin was shown and a polyspecific autoreactivity was proposed for these antibodies (Haynes et al., 2005). Although in preliminary experiments we were unable to confirm these data at least for 2F5, the newly induced neutralising antibodies have to be investigated for their ability to bind cardiolipin. Most importantly, using a p15E/gp41 hybrid protein, antibodies of the 2F5/4E10-type neutralising laboratory and primary strains of HIV-1 were easily induced, suggesting that they do not represent autoimmune antibodies and that the conformation of the antigen is of great importance. As gp41 derived peptides or gp41 proteins were not able to induce neutralising antibodies against HIV-1 (Tian et al., 2001; Lu et al., 2000; McGaughy et al., 2004), it has to be assumed that the conformation of the hybrid antigen allows the induction of such antibodies. However it remains unclear if the neutralising activity depends on a single antibody population. At least one of the monoclonal antibodies generated here (chapter 3.7, mAb3E4E3) from a rat with a HIV-1 neutralising serum recognises an epitope near by the main serum epitope on gp41, but does not show any neutralisation of the virus. These findings might offer a new way for proceeding towards an HIV-1 vaccine based on the induction of broadly neutralising antibodies.

5. Summary

The development of an effective HIV vaccine is more than 20 years after the first description of the virus inducing AIDS one of the major targets in international health efforts. Conventional vaccine strategies including attenuated whole virus vaccines or recombinant viral proteins failed due to safety concerns or ineffective immunological responses. Thus the induction of neutralising antibodies against the HIV-1 transmembrane envelope protein gp41 became a major challenge in HIV vaccine development since it is highly conserved within different HIV-1 subtypes and bears epitopes recognised by the broadly neutralising monoclonal antibodies 2F5 and 4E10. Both antibodies were isolated from humans and showed a protection against HIV-1 of humans and primates in passive immunisation studies. However, until today the induction of such antibodies by active immunisation with a variety of antigen constructs in different animal models, including primates, has not been successful.

In this study presented here the aim was to complete a model for the humoral immune response against the transmembrane envelope protein (TM) of retroviruses in order to evolve a strategy for a successful immunisation against HIV-1 gp41. Therefore the immune response against the TM protein p15E of the Feline Leukaemia virus A (FeLV subtype A), a gammaretrovirus, was characterised in different species including cats as its natural host. Two immune dominant epitope regions (E1 and E2) were determined in the ectodomain of FeLV-A p15E shown to be essential for the neutralisation of the virus *in vitro*. Epitopes within this region detected by antibodies from neutralising immune sera were not only localised similar to those of 2F5 and 4E10 in gp41 but also showed a partial homology to the 4E10 epitope. Further more it was observed that immunisation of cats with the FeLV-A p15E can protect them efficiently from a FeLV-A infection showing for the first time the induction of protective immunity against a retrovirus by immunisation with its transmembrane protein. It was also demonstrated that a protective immunity induced by the unglycosylated FeLV-A surface protein p45 or by the transmembrane protein p15E does not require sterilising immunity.

Based on this FeLV-A p15E model we generated two hybrid constructs, consisting of a p15E backbone and the C-terminal part of HIV-1 gp41 and analysed the humoral immune response against these constructs in the laboratory rat model. By one of these constructs neutralising antibodies specific for HIV-1 gp41 were reproducible induced against the homologous HIV-1 strain as well as against HIV-1 primary isolates. Neutralisation titres similar to those observed for sera from rats immunised with FeLV-A p15E were determined. The epitopes from HIV-1 neutralising antibodies were located within or near by the 2F5 and the 4E10 epitopes. As gp41 derived peptides or gp41 proteins are not able to induce neutralising antibodies against HIV-1, it can be assumed that the conformation of the hybrid antigen must be responsible for this effect. This newly generated antigen might be used as a potential candidate in HIV-1 vaccine studies.

6. Deutschsprachige Zusammenfassung

Mehr als 20 Jahre nach der Entdeckung des Erregers der zu der Immunschwächekrankheit AIDS führt, ist es trotz verstärkter internationaler Bemühungen nicht gelungen einen erfolgreichen Impfstoff gegen das HI-Virus zu entwickeln.

Die Aminosäuresequenz des transmembranen Hüllproteins gp41 von HIV-1 beinhaltet zwei Epitope die von den monoklonalen Antikörpern 2F5 und 4E10 erkannt werden. Diese beiden Antikörper wurden aus HIV-1 infizierten Patienten isoliert und zeigen ein subtypübergreifendes HIV-1 Neutralisationsspektrum. Bis zum heutigen Zeitpunkt ist es nicht gelungen Antikörper mit einer entsprechenden Spezifität durch aktive Immunisierung zu induzieren. Bedingt durch den hohen Konservierungsgrad der Aminosäuresequenz in verschiedenen HIV-1 Subtypen stellt das gp41 Protein ein viel versprechendes Ziel in der HIV-Impfstoffentwicklung dar.

In der vorliegenden Dissertationsarbeit ist die humorale Immunantwort gegen das transmembrane Hüllprotein p15E des γ-Retrovirus FeLV-A (Felines Leukämie Virus Subtyp A) in verschiedenen Spezies charakterisiert worden. Dabei konnten zwei immundominante Epitopregionen beschrieben werden (E1 und E2), die von virusneutralisierenden Antikörpern erkannt werden. Eine dieser Epitopregionen ist, ähnlich dem 2F5 und dem 4E10 Epitop in gp41 Protein, im C-terminalen Bereich des transmembranen Hüllproteins lokalisiert. Für das E2 Epitop von FeLV-A p15E konnte eine partielle Sequenzhomologie mit dem 4E10 Epitop nachgewiesen werden. Weiterhin konnte gezeigt werden, dass die Immunisierung von Katzen mit FeLV-A p15E vor einer produktiven Infektion schützen kann und dass hier die Induktion einer protektiven Immunität nicht von einer sterilisierenden Immunität abhängig ist. Diese Arbeit ist die erste Dokumentation die den Schutz vor einer produktiven retroviralen Infektion durch Immunisierung mit dem entsprechenden transmembranen Hüllprotein zeigt.

Auf der Grundlage des erarbeiteten Modells der humoralen Immunantwort gegen das p15E Protein von FeLV-A wurden zwei Hybridkonstrukte exprimiert, die auf der Aminosäuresequenz des p15E Proteins basieren und eine C-terminale Aminosäuresequenz mit den Epitopen von 2F5 und 4E10 aus HIV-1 gp41 enthalten. Durch die Immunisierung von Ratten mit einem der beiden Konstrukte konnten HIV-1 neutralisierende Antikörper gegen den homologen Virustyp, sowohl als auch gegen Primärisolate induziert werden. Die Titer der HIV-1 neutralisierenden Antikörper entsprachen denen, die mit FeLV-A p15E in Ratten induziert werden konnten. Die Antikörper in den entsprechenden Immunseren erkannten Epitope in der gp41 Aminosäuresequenz, die entweder kurz vor, oder innerhalb der Epitope von 2F5 oder 4E10 lokalisiert waren. Bedingt durch die Tatsache, dass in der Vergangenheit weder durch Immunisierungen mit gp41-abgeleiteten Peptiden, noch mit gp41-Proteinen von HIV-1, neutralisierende Antikörper induziert werden konnten, kann davon ausgegangen werden, dass die Konformation des hier beschriebenen Hybridproteins dafür verantwortlich ist. Das hier beschriebene Hybridprotein könnte die Grundlage für einen effizienten HIV-1 Impfstoff darstellen.

6. References

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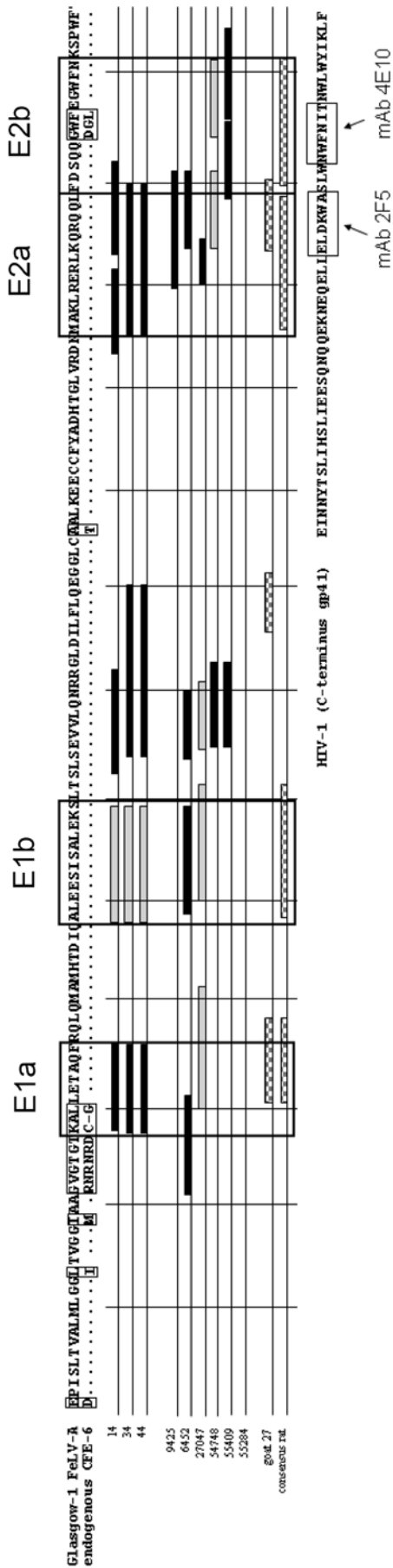
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7. Appendix

7.1 Chapter 3.3.4, figure 25

Figure 25 Epitope mapping using sera from immunised and FeLV-infected cats. Summary of the epitopes identified recognised by each serum. Sequences corresponding to the recombinant p15E of FeLV-A, strain Glasgow 1, and the corresponding sequence of an endogenous p15E are given at the top. Cats 14, 34 and 44 were immunised with p15E while the others are representative of FeLV-infected cats. Strong epitopes are marked in black, weak epitopes in grey. Common 2 groups of epitopes are framed (E1a, E1b, E2a, E2b). In addition, epitopes recognised by the serum from goat 27, immunised with p15E, and consensus epitopes recognised by 8 rats immunised with p15E are shown (hatched)6. For comparison, the C-terminal part of the HIV-1 transmembrane envelope protein gp41 and the localisation of epitopes recognised by two monoclonal antibodies (mAb) broadly neutralising HIV-1, (2F5 and 4E10) are shown (framed).

7.2 Primer and probes (Sigma-Genosys, Steinheim, Germany)

1. FeLV-A p15E forward:
   ```
   5´-GCGGATCCCTTGAAACAGCCCAGTTCAGACAA-3´
   ```

2. FeLV-A p15E reverse:
   ```
   5´-CGGAATTCCCAGGGGGACTTGTTGAACCATCC-3´
   ```

3. p4:
   ```
   5´-ATGCGGCCGCGCAACATTCTTCTTTCAATGCGGCACAGAGTTCAGAAAGGGAGGT-
   3´
   ```

4. p5:
   ```
   5´-GCGCGGCCGCAGACTAAAACAGCGGCAACA-3´
   ```

5. p5 hybrid I:
   ```
   5'-ATGCGGCCGCATTAATTGAAGAATCGCAAAA-3'
   ```

6. p5 hybrid II:
   ```
   5'-ATGCGGCCGCAGAAAAGAATGAACAAGAA-3'
   ```

7. FeLV-RTQ:

   ```
   sense primer:
   5´-TCAAGTATGTTCCCATGAGATACAA-3´
   ```

   ```
   antisense primer:
   5´-GAAGGTCGAACTCTGGTCAACT-3´
   ```

   ```
   FeLV-A probe:
   5`-6Fam-TTAAGCACCTGGGCCCCGGC-Dabcyl-3´
   ```

8. HIV-RTQ:

   ```
   68i: SK68i M:
   5'-GGARCAGCIGGAAGCACIATGG-3´
   ```

   ```
   69i: SK69i M:
   5´-CCCCAGACIGTGAGITICAACA-3´
   ```

   ```
   HIV-1 probe:
   5´- 6Fam-TGACGCTGACGGTACAGGCCAGAC-Dabcyl-3´
   ```

9. PERV-RTQ:

   ```
   PERV real s:    5'-TCCAGGGCTCATAATTTGTC-3´
   ```

   ```
   PERV real_as:  5'-TGATGGCCATCCAACATCGA -3´
   ```

   ```
   PERV probe:     5'-6Fam-AGAAGGGACCTTGGCAGACTTTCT –Dabcyl-3'
   ```

7.3 Amino acid and nucleotide sequences of recombinant constructs without CBP

1. FeLV-A p15E ectodomain:

CTTGAAACAGCCCAGTTCAGACAACTACAAATGGCCATGCACACAGACATCCAGGCCCTAGA
AGAATCAATTAGTGCCTTAGAAAAGTCCCTGACCTCCCTTTCTGAAGTAGTCTTACAAAACA
GACGGGGCCTAGATATTCTATTCTTACAAGAGGGAGGGCTCTGTGCCGCATTGAAAGAAGAA
TGTTGCTTCTATGCGGATCACACCGGACTCGTCCGAGACAATATGGCCAAATTAAGAGAAAG
ACTAAAACAGCGGCAACAACTGTTTGACTCCCAACAGGGATGGTTTGAAGGATGGTTCAACA
AGTCCCCCTGGTTT

LETAQFRQLQMAMHTDIQALEESISALEKSLTSLSEVVLQNRRGLDILFLQEGGLCAALKEE
CCFYADHTGLVRDNMAKLRERLKQRQQLFDSQQGWFEGWFNKSPWF

2. FeLV-A ΔISU p15E:

CTTGAAACAGCCCAGTTCAGACAACTACAAATGGCCATGCACACAGACATCCAGGCCCTAGA
AGAATCAATTAGTGCCTTAGAAAAGTCCCTGACCTCCCTTTCTGAACTCTGTGCCGCATTGA
AAGAAGAATGTTGCGCGGCCGCAAGACTAAAACAGCGGCAACAACTATTTGACTCCCAGCAG
GGATGGTTTGAAGGATGGTTCAACAAGTCCCCCTGGGAATTCTAG

LETAQFRQLQMAMHTDIQALEESISALEKSLTSLSELCAALKEECCAAARLKQRQQLFDSQQ
GWFEGWFNKSPWF

3. p15E/gp41 hybrid protein I:

CTTGAAACAGCCCAGTTCAGACAACTACAAATGGCCATGCACACAGACATCCAGGCCCTAGA
AGAATCAATTAGTGCCTTAGAAAAGTCCCTGACCTCCCTTTCTGAACTCTGTGCCGCATTGA
AAGAAGAATGTTGCGCGGCCGCATTAATTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAA
CAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAACATAACAAATTG
GCTGGAATTCTAGA

LETAQFRQLQMAMHTDIQALEESISALEKSLTSLSELCAALKEECCAAALIEESQNQQEKNE
QELLELDKWASLWNWFNITNWLEF

4. p15E/gp41 hybrid protein II:

CTTGAAACAGCCCAGTTCAGACAACTACAAATGGCCATGCACACAGACATCCAGGCCCTAGA
AGAATCAATTAGTGCCTTAGAAAAGTCCCTGACCTCCCTTTCTGAACTCTGTGCCGCATTGA
AAGAAGAATGTTGCGCGGCCGCAGAAAAGAATGAACAAGAATTATTGGAATTAGATAAATGG
GCAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCTG

LETAQFRQLQMAMHTDIQALEESISALEKSLTSLSELCAALKEECCAAAEKNEQELLELDKW
ASLWNWFNITNWL

8. List of publications

(1)Langhammer, S., Fiebig, U., Kurth, R., Denner, J. Neutralising antibodies against the transmembrane protein of feline leukaemia virus (FeLV). Vaccine 2005; 23:3341-3348.

(2)Langhammer, S., Fiebig, U., Kurth, R., Denner, J. Jahrestagung Gesellschaft für Virologie, 03/16.-03/19/2005, RET 28, 113

(3)Langhammer, S., Hübner, J., Kurth, R., Denner, J. Antibodies neutralising feline leukemia virus (FeLV) in cats immunised with the transmembrane envelope protein p15E: Absence of tolerance against homologous endogenous sequences. Immunology, in press

(4)Langhammer, S., Fiebig, U., Kurth, R., Denner, J. Induction of neutralising antibodies against the transmembrane envelope protein gp41 of the human immunodeficiency virus HIV-1, The Journal of Immunology, submitted

(5)Langhammer, S., Hübner, J., Kurth, R., Denner, J. Protective immunity against a retrovirus by immunisation with its transmembrane envelope protein. Immunity, submitted

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