

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).

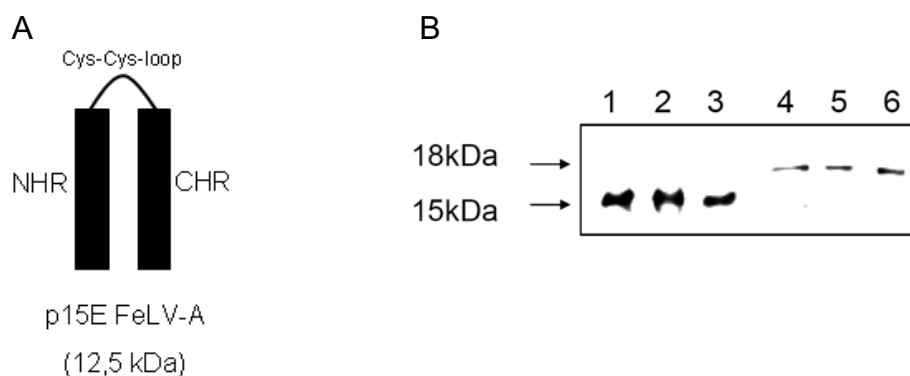


Figure 8 (A) Schematic model of the FeLV-A p15E ectodomain without CBP. Indicated are the cysteine loop (Cys-Cys-loop) and the N- and C-terminal helix regions (NHR, CHR). **(B)** Western blot analysis of goat antiserum specific for FeLV-A p15E (1, 4), of affinity purified antibodies (2, 5) and of a goat 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.

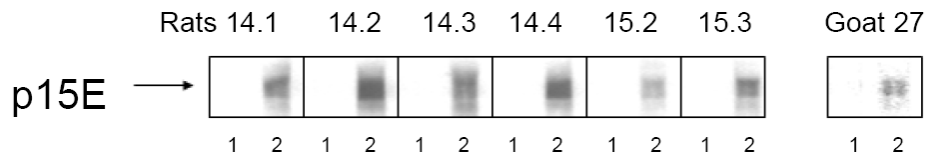


Figure 9 Western blot analysis of rat and goat antisera following immunisation with FeLV-A p15E. The antigen used was the same recombinant p15E ectodomain used for immunisation. The number of the serum tested is indicated at the top. Lane 1 shows the preimmune serum, lane 2 the corresponding immune serum.

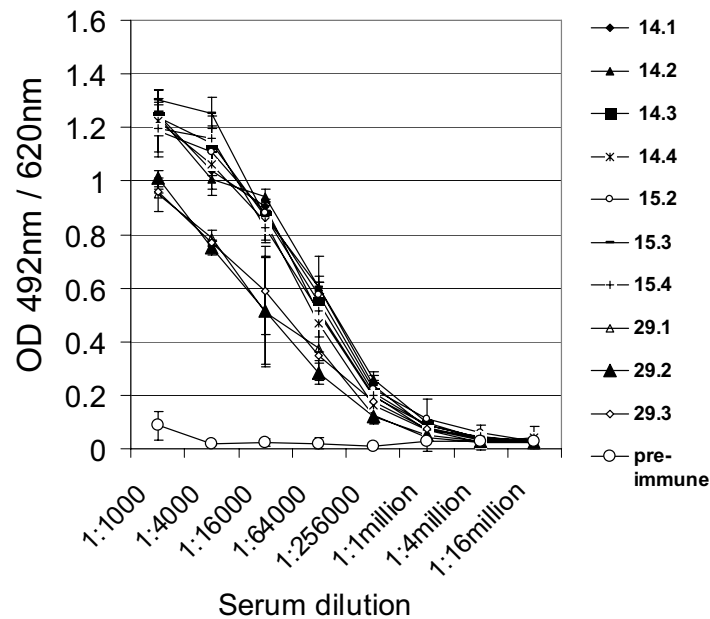


Figure 10 Determination of the ELISA titres of rat and goat antisera using the recombinant ectodomain of p15E as antigen. Immune sera and preimmune sera are indicated on the right.

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.

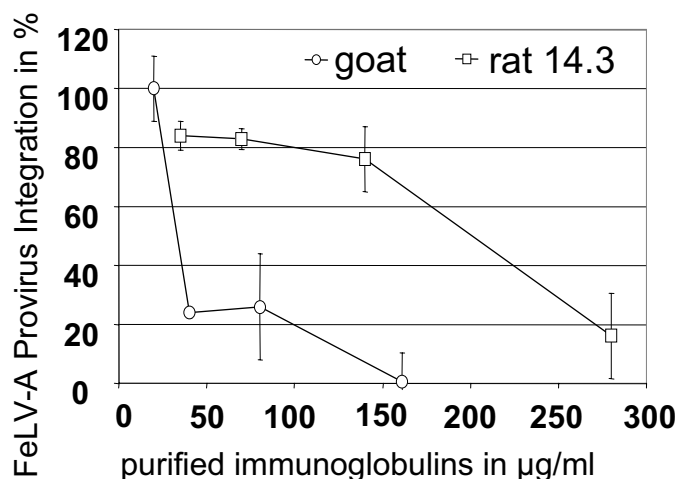


Figure 11 Neutralisation of FeLV-A Glasgow strain by p15E affinity purified immunoglobulins from goat serum 27 and protein G purified IgG from rat serum 14.3. Infection was measured by real time PCR detecting provirus integration and indicated as percentage of the signal obtained with infected cells 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.

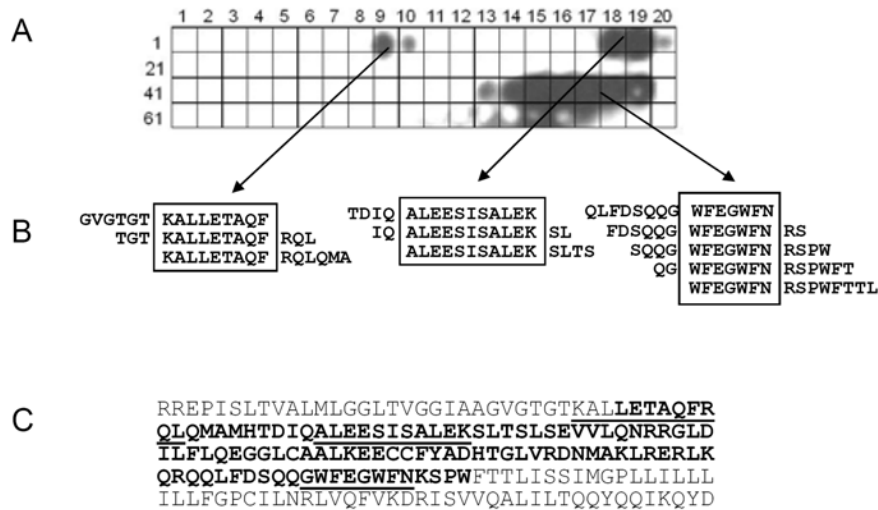


Figure 12 Epitope mapping using neutralising rat serum 15.3 specific for FeLV-A p15E. The result of the ECL dot blot using overlapping peptides is given in **A**, followed by the sequence alignment of the mapped peptides in **B**. In **C** the sequence of FeLV-A p15E is given, the sequence of the recombinant 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.

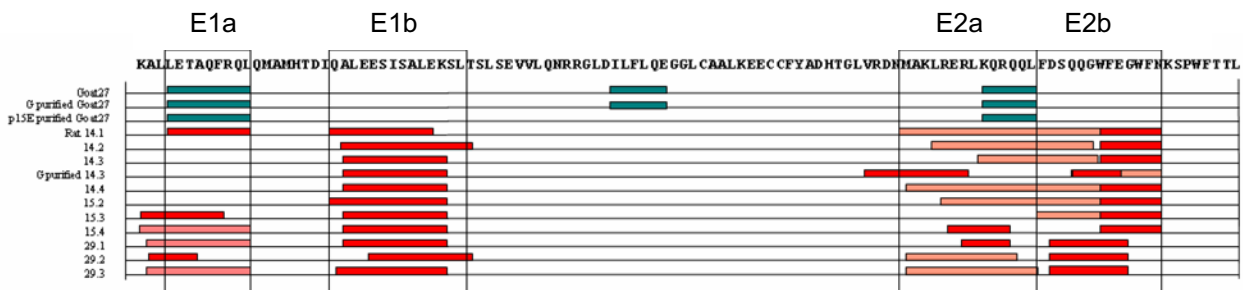


Figure 13 Results of the epitope mapping of different rat (marked in red) and goat (marked in green) antisera specific for p15E as well as of affinity purified IgG from two sera. The sequence of FeLV-A p15E is given in part, epitope regions are framed and indicated at the top.

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).

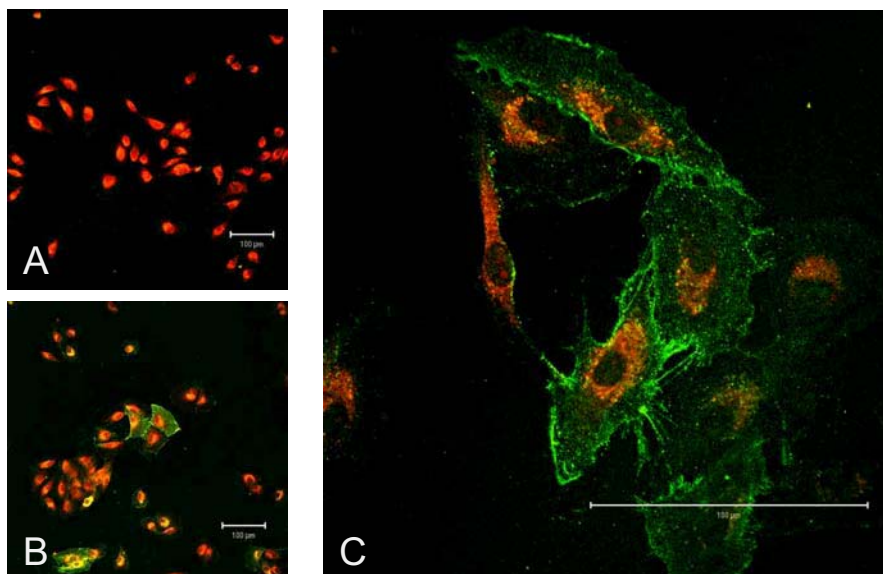


Figure 14 Indirect immunofluorescence, visualised by confocal laser microscopy, using IgG purified by p15E affinity chromatography from goat serum specific for p15E and FITC conjugated anti-goat IgG Ab. FITC staining was measured at 488nm and unspecific fluorescence measured at 543nm was subtracted. Uninfected FEA cells are shown in (A), FeLV-A producing FEA cells are shown in (B) and (C). The white bars indicate 100 μ m.

3.2 Induction of neutralising antibodies against FeLV-A Δ ISU 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 Δ ISU 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).

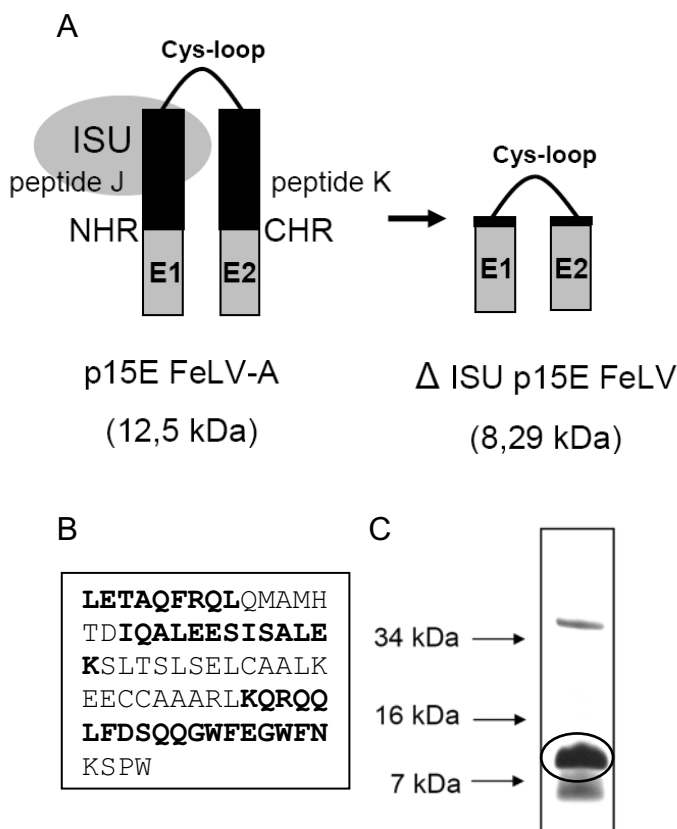


Figure 15 Characterisation of the FeLV-A Δ ISU p15E protein. **(A)** Schematic presentation of the p15E ectodomain of FeLV and the modified Δ ISU p15E protein. The localisation of the isu domain, the peptides J and K, the cystein loop as well the N- and C-helix regions (NHR, CHR) and the epitope regions (E1, E2) are indicated. **(B)**, Amino acid sequence of FeLV-A Δ ISU p15E. Identified epitope regions are printed in bold. **(C)** SDS-page analysis of semi-purified Δ ISU 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 $\geq 1:16$.

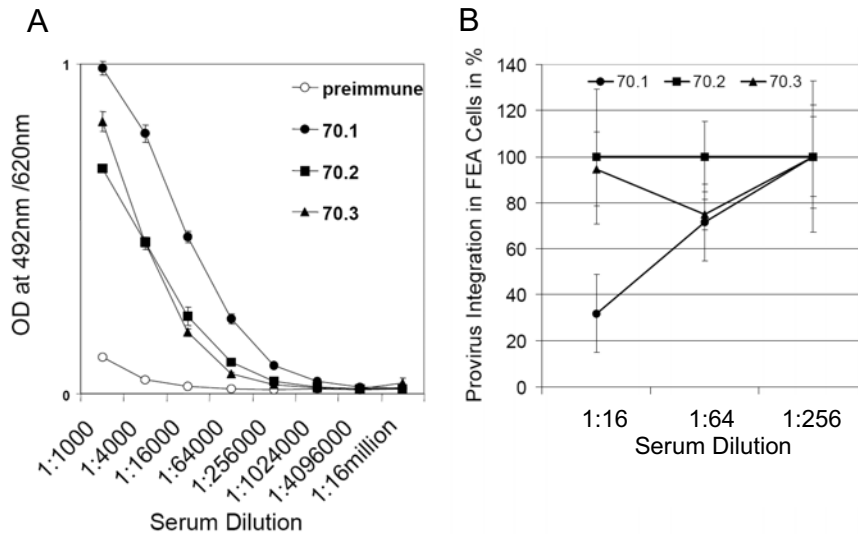


Figure 16 Characterisation of antisera from rats 70.1, 70.2 and 70.3. (A) Determination of the ELISA titres using the recombinant ectodomain of p15E as antigen. Immune sera and preimmune sera are indicated on the right. (B) Neutralisation of FeLV-A Glasgow strain at a serial dilution of antisera (1:16-1:256) 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).

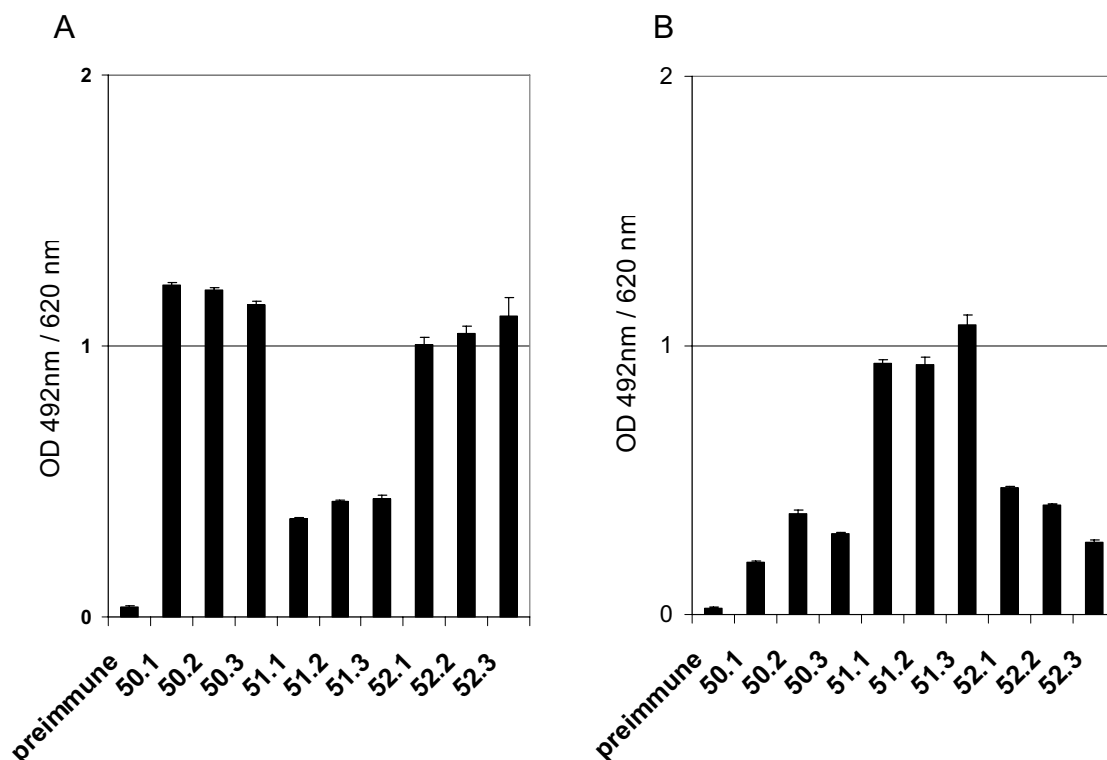


Figure 18 ELISA reactivity of rat antisera induced by immunisation with 500µg p15E alone (group 50), with 100µg p45 alone (group 51) and with a combination of both antigens (group 52). As antigen in the ELISA FeLV-A p15E (A) or Leucogen (B) were used.

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 500 μ g p15E to only 100 μ g 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 500 μ g p15E (group 55) was compared with injection of 100 μ g 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, 100 μ g Leucogen were injected together with 500 μ g p15E (group 54) or together with 100 μ g 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: 500 μ g p15E

56: 100 μ g p45

57: 100 μ g p15E

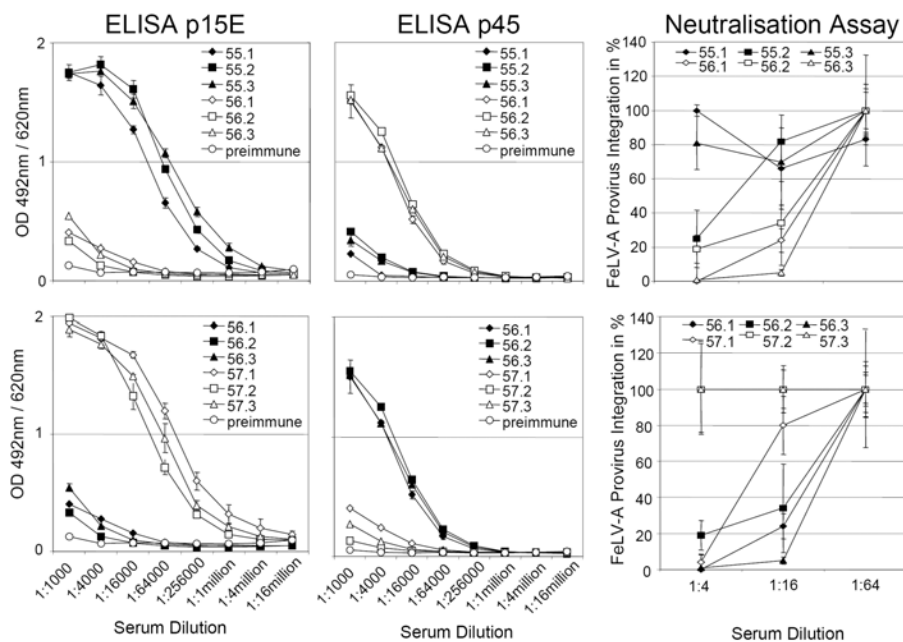


Figure 19 ELISA reactivity and neutralising activity of rat antisera induced by immunisation with 500 μ g p15E alone (group 55), with 100 μ g p15E alone (group 57) and with 100 μ g Leucogen alone (group 56). As antigen in the ELISA FeLV-A p15E or Leucogen were used.

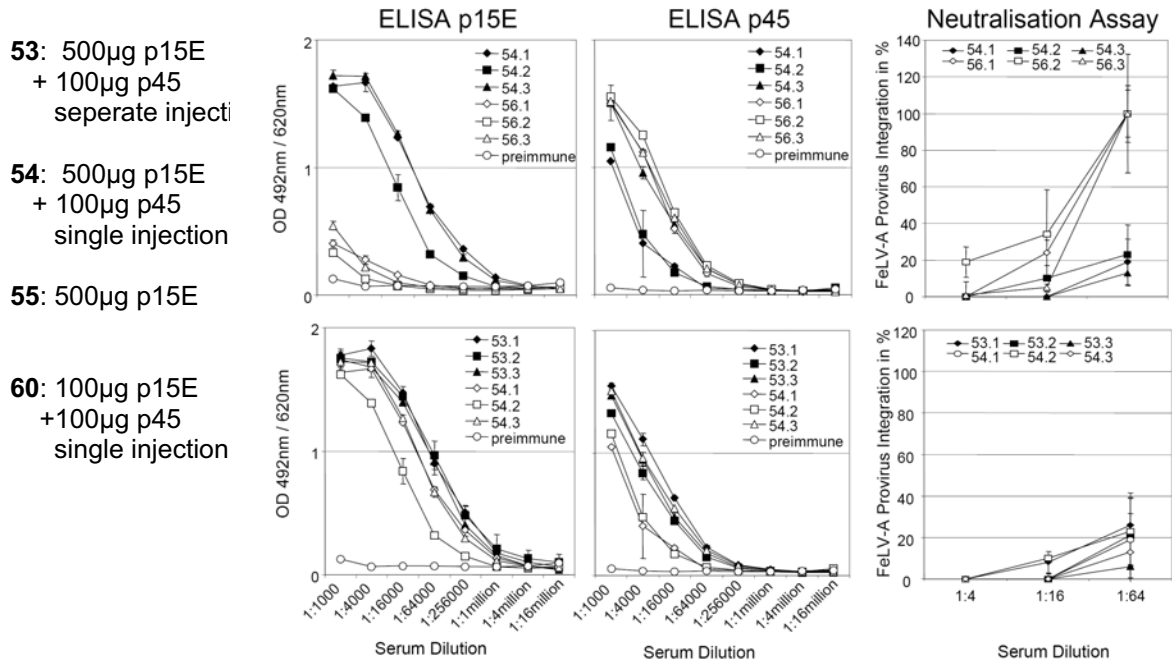


Figure 20 ELISA reactivity and neutralising activity of rat antisera induced by Immunisation with 500µg p15E in combination with 100µg Leucogen in a single injection site (group 54) or in two different injection sites (group 53) and with Leucogen alone (group 56). As antigen in the ELISA FeLV-A p15E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

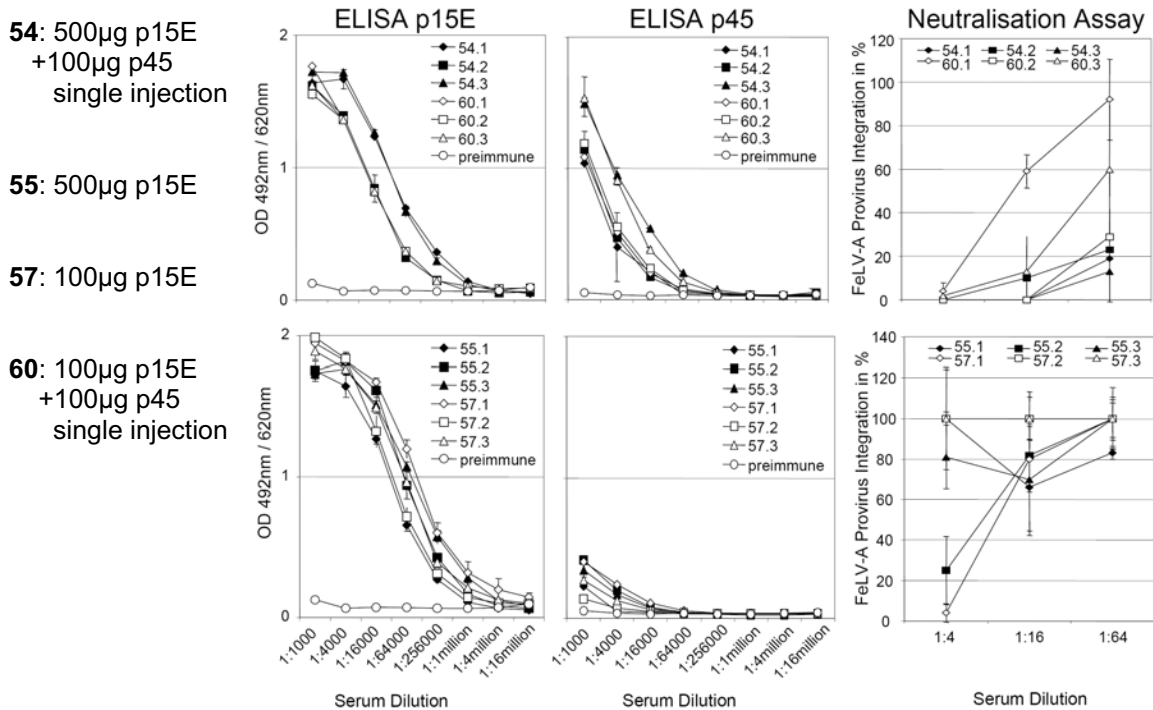


Figure 21 ELISA reactivity and neutralising of rat antisera induced by Immunisation with 500µg p15E in combination with 100µg Leucogen in a single injection site (group 54), with 100µg p15E in combination with 100µg Leucogen in a single injection site (group 60), with 500µg p15E alone (group 55) and with 100µg p15E alone (group 57). As antigen in the ELISA FeLV-A 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 (FDSQQGWFEQWFN). In contrast to the previous study (chapter 3.1), the E2a epitope was better defined (KQRQQLF instead of MAKLRERLQQRQQL). 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.

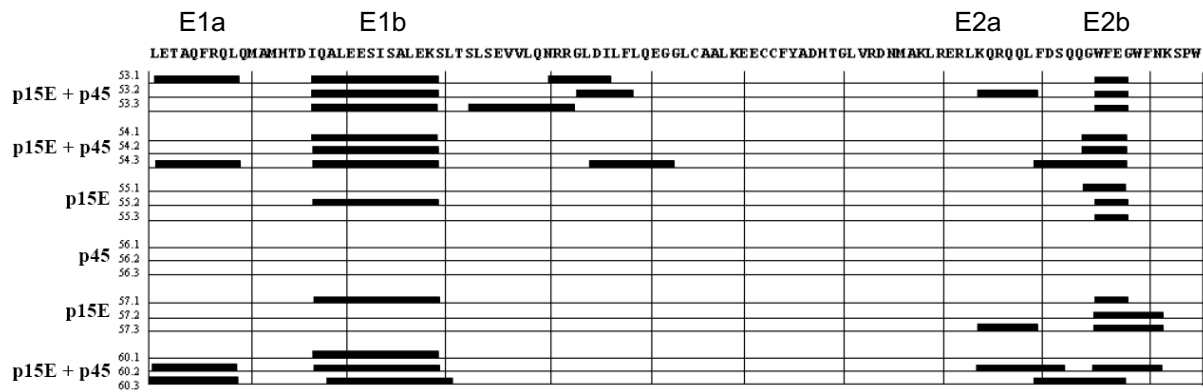


Figure 22 Summary of the specific epitope mapping of all antisera obtained after immunisation with p15E, Leucogen or both, p15E and Leucogen. The sequence of the FeLV-A p15E ectodomain and the animal number and the antigen(s) used for immunisation are given, epitopes are framed.

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	1x10 ⁶	6.4x10 ⁴	1:64	+	+	-	+
	53.2	500	100	Two separate	Montanide	1x10 ⁶	6.4x10 ⁴	1:64	-	+	+	+
	53.3					1x10 ⁶	6.4x10 ⁴	1:64	-	+	-	+
54	54.1				LA	1x10 ⁶	1.6x10 ⁴	1:64	-	+	-	+
	54.2	500	100	Single	LA	2.56 x10 ⁵	1.6x10 ⁴	1:64	-	+	-	+
	54.3				Montanide	1x10 ⁶	6.4x10 ⁴	1:64	+	+	-	+
55	55.1				Montanide	1x10 ⁶	-	0	-	-	-	+
	55.2	500	-	-	Montanide	4x10 ⁶	-	1:16	-	+	-	+
	55.3				Montanide	4x10 ⁶	-	0	-	-	-	+
56	56.1				LA	-	6.4x10 ⁴	1:16	-	-	-	-
	56.2	-	100	-	LA	-	6.4x10 ⁴	1:16	-	-	-	-
	56.3				Montanide	-	6.4x10 ⁴	1:16	-	-	-	-
57	57.1				Montanide	4x10 ⁶	-	1:16	-	+	-	+
	57.2	100	-	-	Montanide	1x10 ⁶	-	0	-	-	-	+
	57.3				Montanide	4x10 ⁶	-	0	-	-	+	+
60	60.1				LA	2.56 x10 ⁵	1.6x10 ⁴	1:16	-	+	-	-
	60.2	100	100	Single	LA	2.56 x10 ⁵	1.6x10 ⁴	1:64	+	+	+	+
	60.3				LA	2.56 x10 ⁵	6.4x10 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.

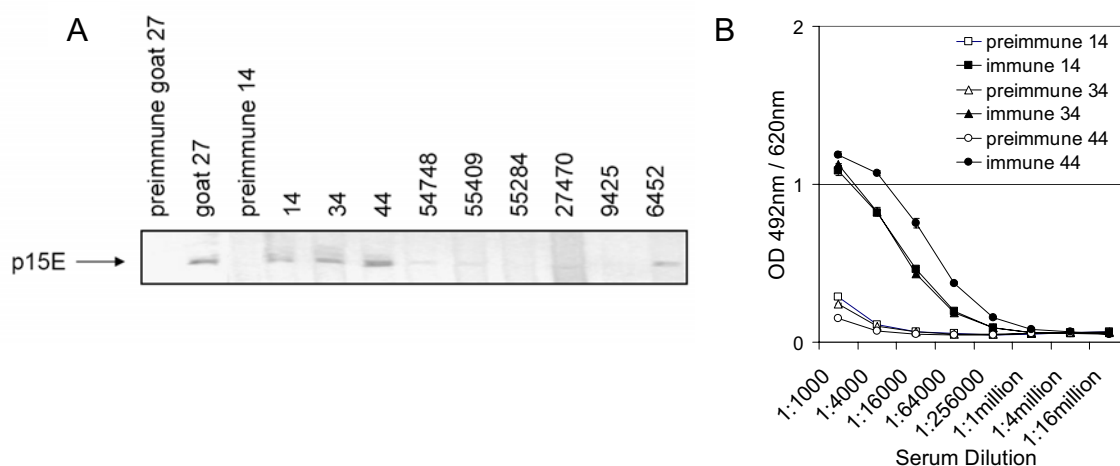


Figure 23 (A) Western blot analysis of cat antisera following immunisation with FeLV-A p15E (14, 34 and 44) and of sera from FeLV-infected cats (54748-6452). The same recombinant p15E used for immunisation was used as antigen. Lane 1 shows the preimmune serum of goat 27, lane 2 the corresponding immune 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 (2.56×10^5 after the first immunisation and 1×10^6 after the boost). The sera from cats 14 and 34 showed titres of 6.4×10^4 that increased to 2.56×10^5 after the boost immunisation. In contrast, sera obtained from FeLV-infected housecats had only titres between 1×10^3 and 4×10^3 (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.

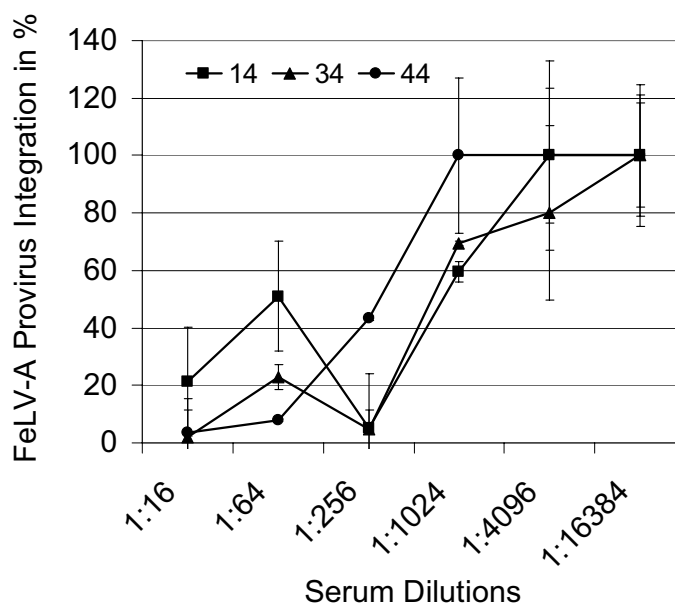


Figure 24 Neutralising activity of cat antisera after the second immunisation with 500µg p15E. Infection was measured as provirus integration by real-time PCR. Percent of provirus integration was obtained by comparing antisera with the corresponding preimmune sera.

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, MAKLRERLKQRQLF, 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 (FDSQQGWFEQWFN). 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.

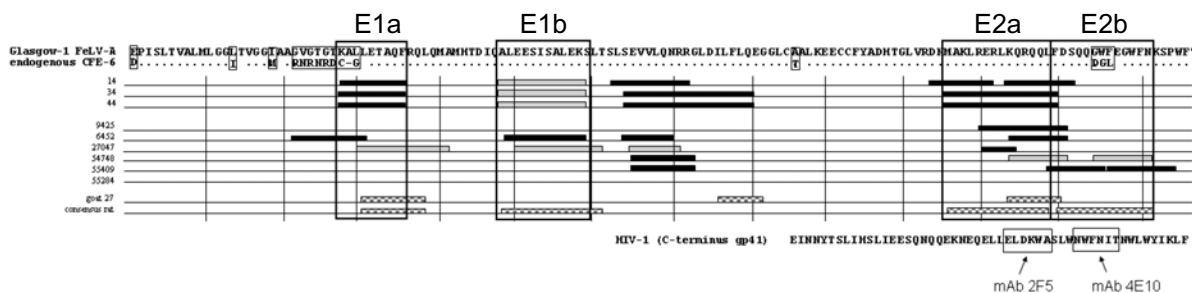


Figure 25 Epitope mapping using sera from immunised and FeLV-infected cats. Summary of the epitopes identified recognised by each serum. Sequences corresponding to the recombinant p15E of FeLV-A, strain Glasgow 1, and the corresponding sequence of an endogenous p15E are given at the top. Cats 14, 34 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). 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.

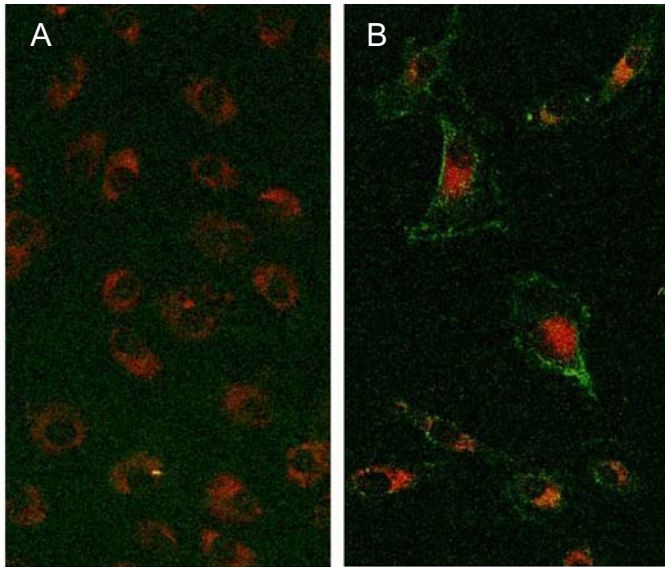


Figure 26 Indirect immunofluorescence, visualised by confocal laser microscopy, using antiserum from cat 44 (immunised with p15E) and FITC-conjugated anti-cat IgG. FITC staining was measured at 488nm and unspecific fluorescence measured at 543nm was subtracted. **(A)** Uninfected FEA cells, **(B)** FeLV-A 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	-	+	(6,4x10 ⁴) 2,56 x10 ⁵	(1:256) 1:1024	++	+	++	-
34	p15E	-	+	(6,4x10 ⁴) 2,56 x10 ⁵	(1:256) 1:1024	++	+	++	-
44	p15E	-	+	(2,56 x10 ⁵) 1 x10 ⁶	(1:256) 1:256	++	+	++	-
9425	none	+	-	≤1 x10 ³	1:16	-	-	-/+	-
6452	none	+	+	4 x10 ³	1:256	-	++	++	-
27470	various vaccines	+	-/+	4 x10 ³	1:256	+	+	-/+	-
54748	none	+	+	4 x10 ³	0	-	-	+	+
55409	none	+	-/+	4 x10 ³	1:64	-	-	+	+
55284	none	+	-	≤1 x10 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).

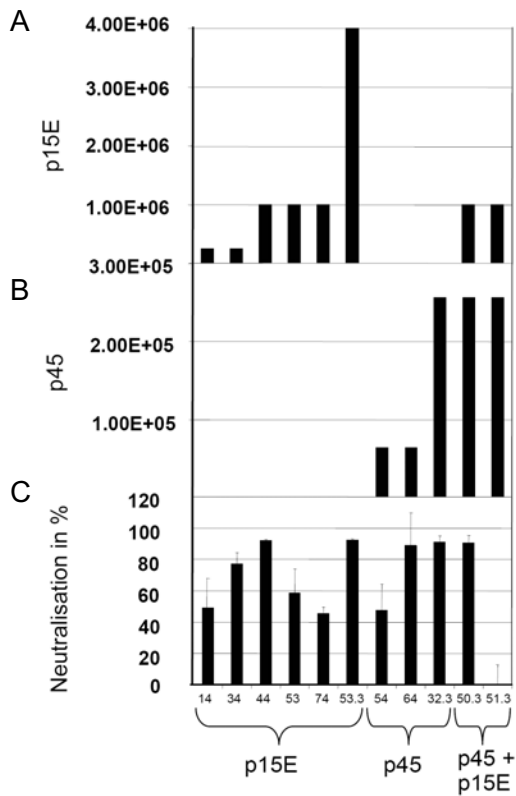


Figure 27 Titres of binding antibodies against p15E (A) and p45 (B) determined in an ELISA with a four fold serial dilution of antisera compared to corresponding preimmune sera. (C) Neutralising activity in percentage obtained for sera from immunisation with p15E, Leucogen or with a combination of both as shown by indicated brackets. The neutralising activity was determined in relative to corresponding preimmune 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.

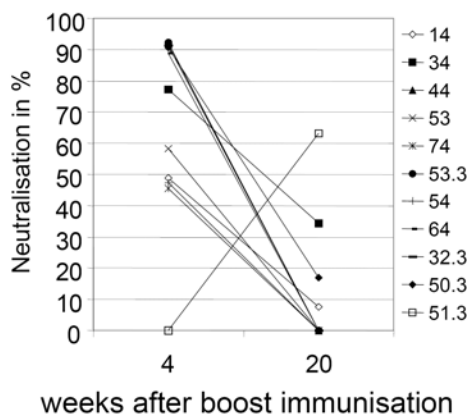


Figure 28 Comparison of neutralising antibodies 4 weeks and 20 weeks after immunisation corresponding to three days before the virus challenge. Shown is the percentage of neutralising activity for sera obtained from immunisation with p15E, Leucogen or with a combination of both as shown by indicated brackets. The neutralising activity was determined in relative to corresponding preimmune 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.

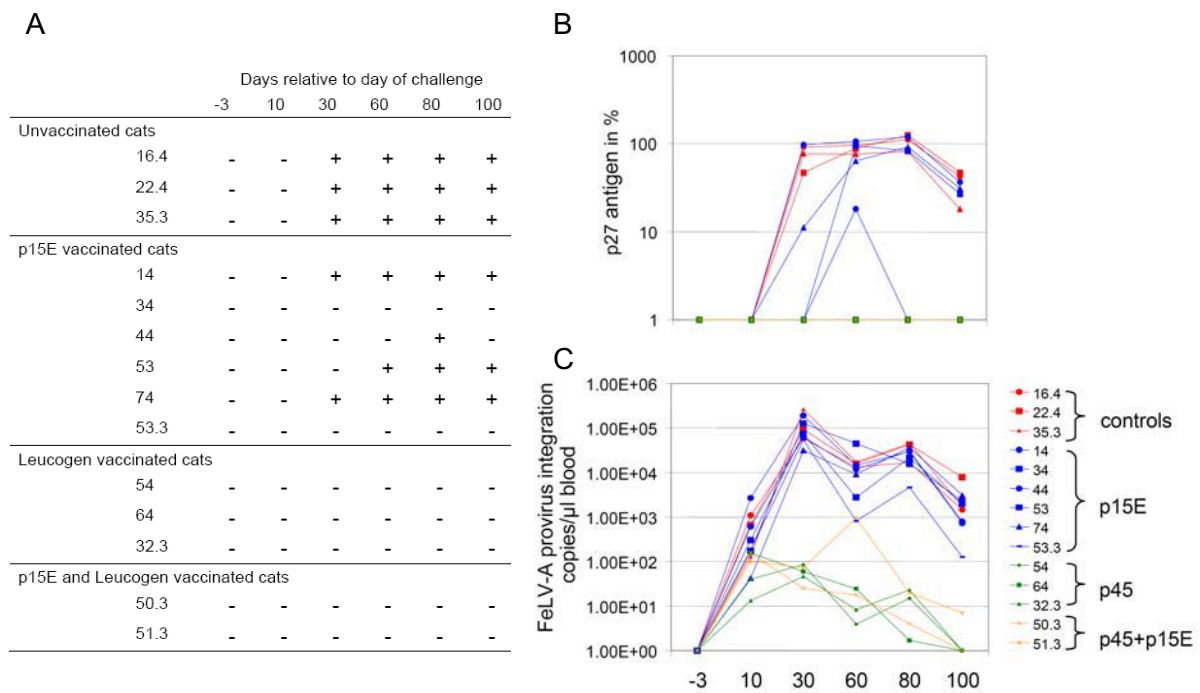


Figure 29 Response of cats to challenge with FeLV (**A**) Summary of presence (+) or absence (-) of p27 antigen from blood samples. (**B**) Kinetics of FeLV provirus integration in copies/ μ l blood measured in non-immunised control animals (red), in animals immunised with p15E (blue), with Leucogen 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/ μ l 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 1.08×10^2 and 1.26×10^2 copies/ μ l blood were observed, increasing up to 5.82×10^4 and 2.66×10^5 copies/ μ l blood at day 30 after challenge. This level decreased to below 1×10^4 copies/ μ l 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 1×10^3 copies/ μ l 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).

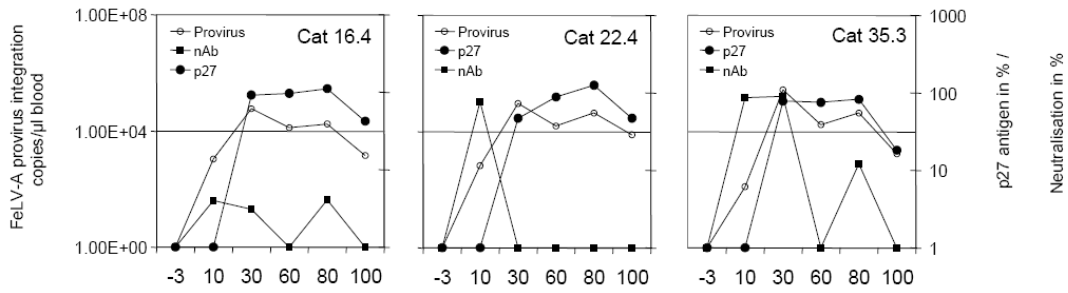
In contrast to the animals of the non-immunised control group and to the animals immunised with p15E alone, low levels of provirus integration were observed in the blood of animals immunised with Leucogen alone or in combination with p15E (Leucogen: 54, 64, 32.3; combination: 50.3, 51.3). Starting with day 10 after challenge the level of provirus integration ranged between 1.32×10^1 and 1.57×10^2 copies/ μ l blood decreasing to 7.05 copies/ μ l 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

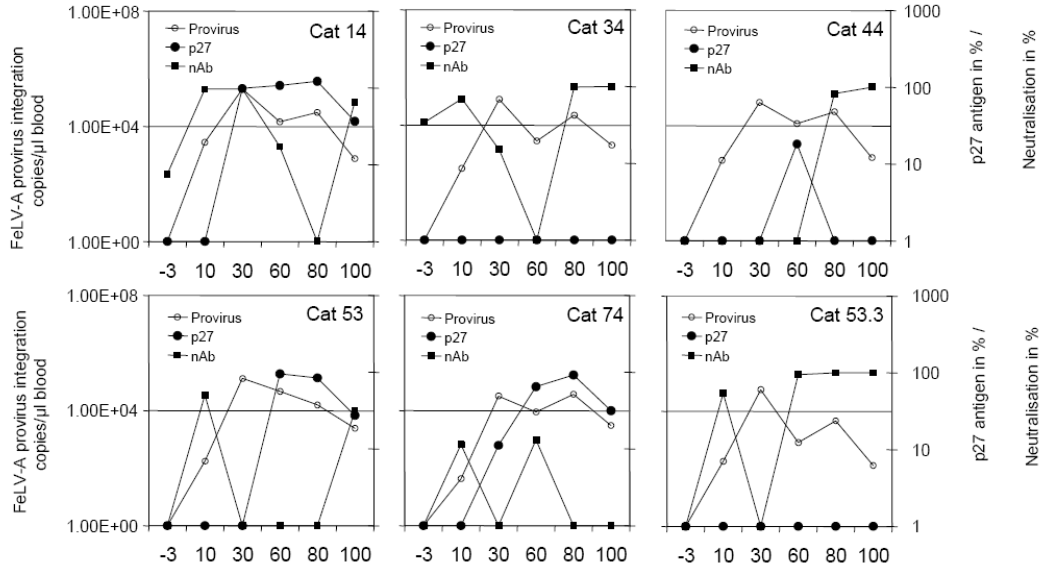
To analyse whether neutralising antibodies represent a correlate for protection, the relationship between provirus load in copies/ μ l blood and the p27 antigen load in percentage on one hand and the neutralising capacity of the serum on the other was investigated.

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.

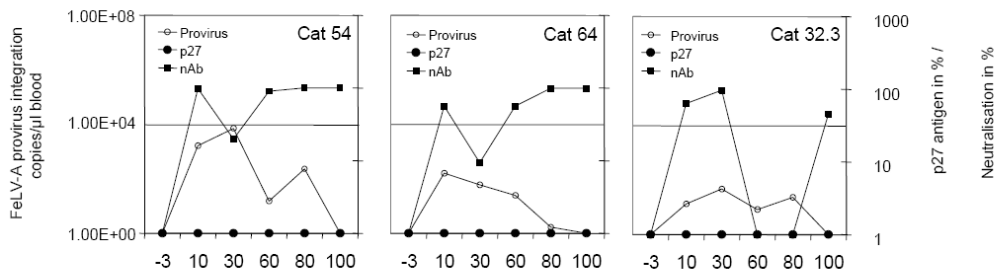
Unvaccinated cats



p15E vaccinated cats



Leucogen vaccinated cats



p15E and Leucogen vaccinated cats

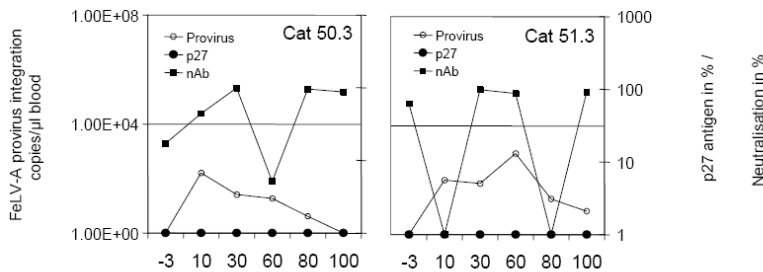


Figure 30 Kinetics of provirus integration in copies/μl blood, prevalence of p27 antigen in percentage and neutralising activity (measured at a serum dilution of 1:100 in % relative to preimmune serum) in cats.

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 Δ ISU 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 boosted with 0.1mg gp41-derived peptide once.

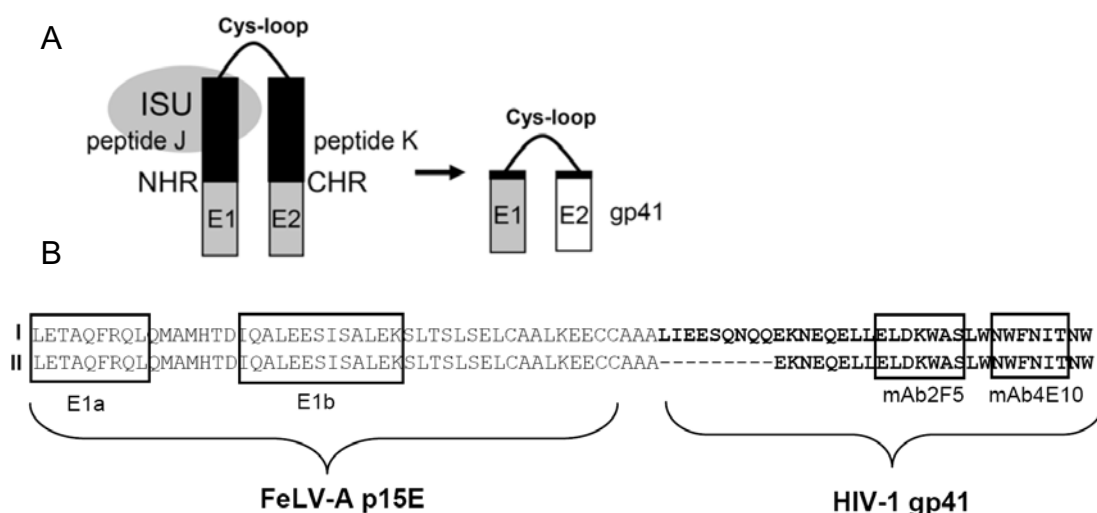


Figure 31 Properties of the p15E/gp41 hybrid proteins I and II. **(A)** Schematic presentation of the p15E ectodomain of FeLV and the modified p15E/gp41 hybrid proteins I and II. The localisation of the isu domain, the peptides J and K, the cystein-cystein loop as well as the N- and C-helix regions (NHR, 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.

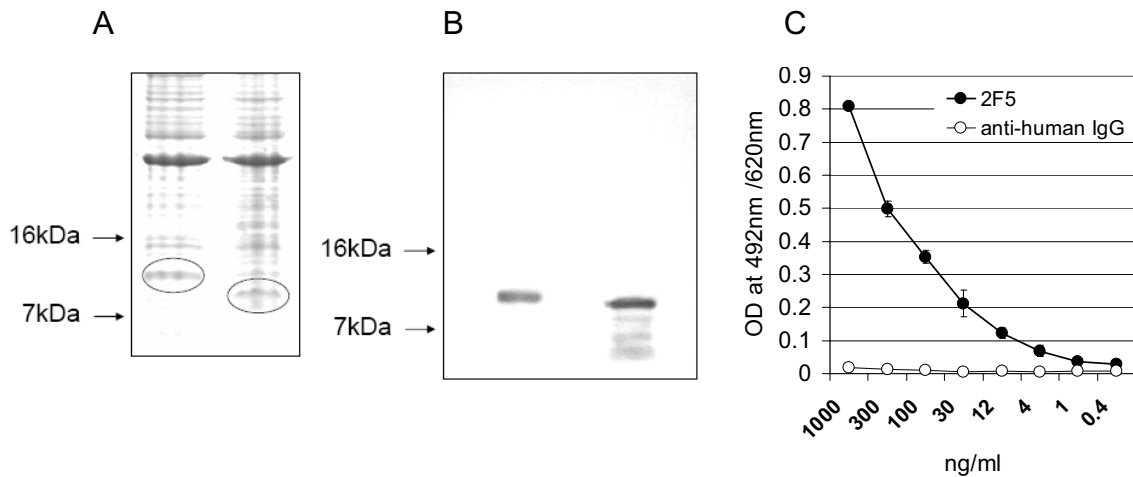
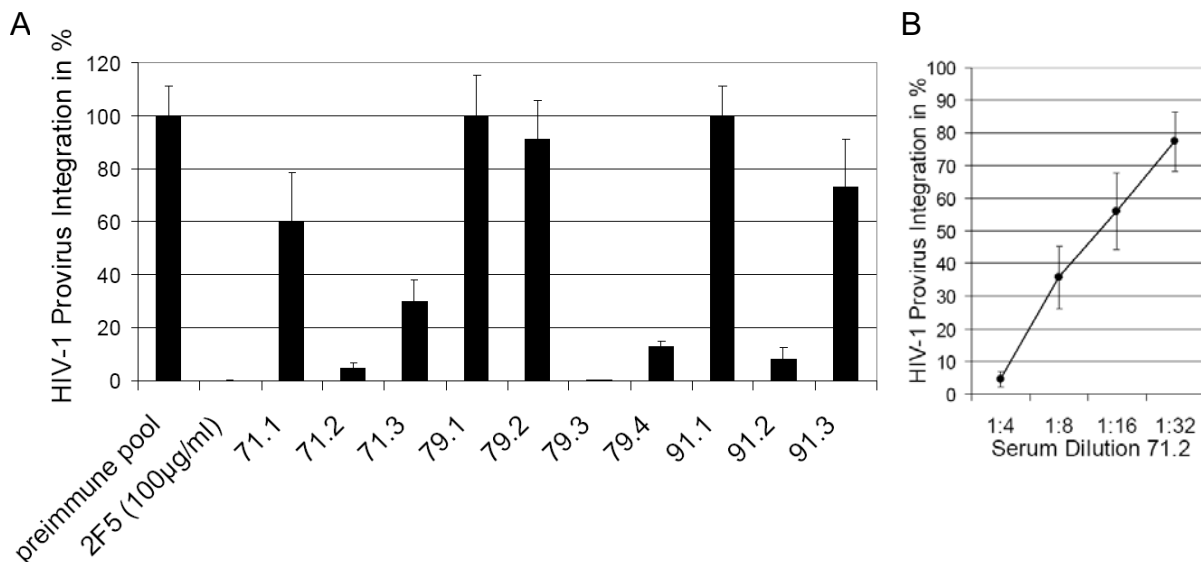


Figure 32 Characterisation of the p15E/gp41 hybrid proteins. **(A)** SDS-Page of p15E/gp41 hybrid I (1) and II (2) indicated in red circles and **(B)** detection of the p15E/gp41 hybrid I (1) and II (2) in a Western blot analysis with mAb 2F5 (0.5mg/ml). 1 μ g of hybrid protein suspension was loaded 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-1IIIIB 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 boosted with the p15E/gp41 hybrid I, 5 rats showed a neutralisation of up to 99% of the HIV-1IIIIB (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).



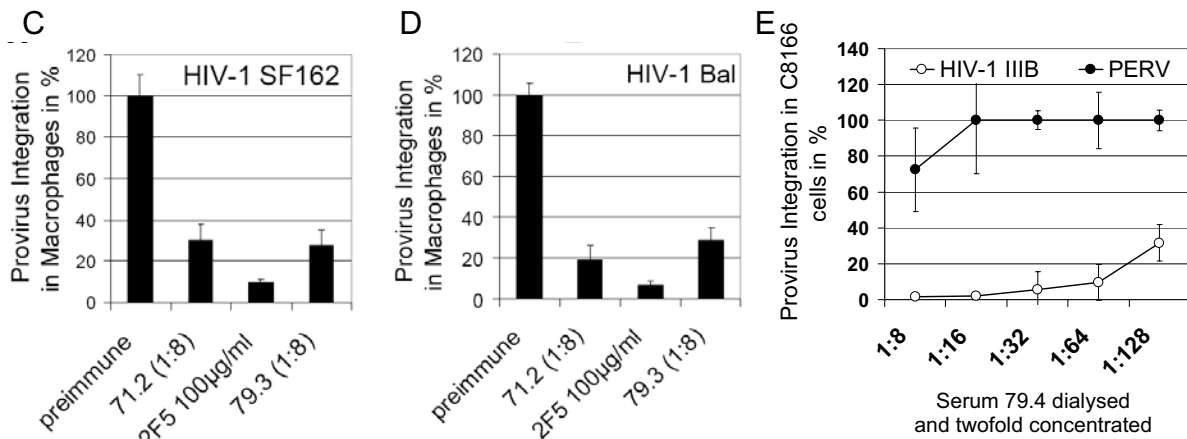


Figure 33 Neutralisation of HIV-1IIIB and of two HIV-1 primary isolates by rat antisera induced by immunisation with the p15E/gp41 hybrid protein I. Virus infection was measured as provirus integration by quantitative real time PCR. The mAb2F5 was used as positive control at a concentration of 100µg/ml. (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 dose-dependent 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 boosted with p15E/gp41 hybrid II (Fig. 34; Tab.6) or immunised with p15E/gp41 hybrid I and boosted 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.

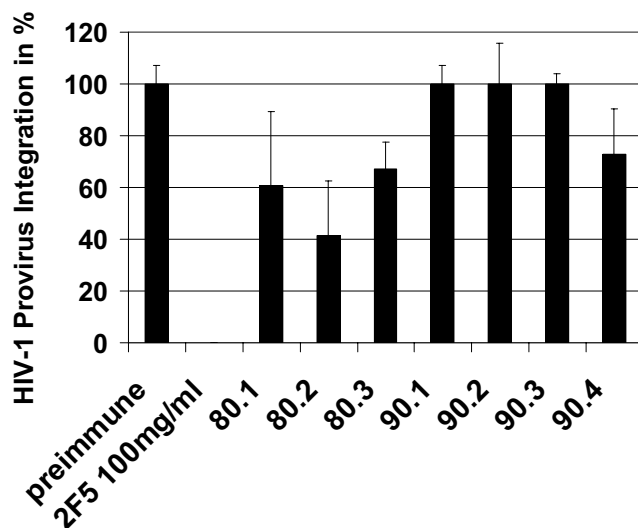


Figure 34 Neutralisation efficacy of HIV-1 IIIB by rat antisera induced by immunisation with p15E/gp41 hybrid I and boosted with gp41-derived peptide E2 (group 80) or by immunisation and boost with the p15E/gp41 hybrid II (group 90). Virus infection was measured as provirus integration by quantitative 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 (QELLELDKW) 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).

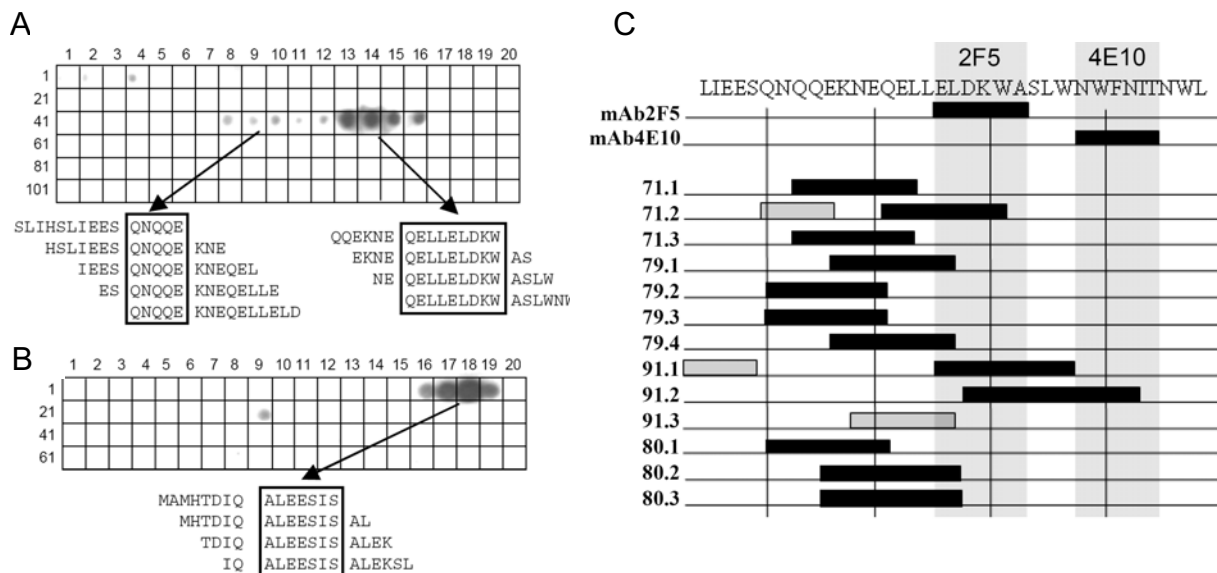


Figure 35 Epitope mapping using pepspot membranes with overlapping peptides and rat sera after immunisation with the p15E/gp41 hybrid proteins. The ECL method was used for detection. **(A, B)** Results obtained with the neutralising rat serum 71.2 using overlapping peptides corresponding to the entire 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	6.4x10 ³	0	0%	EKNEQEELLE
79.2		6.4x10 ³	0	0%	QNQQEKNE
79.3		6.4x10 ³	1:16	99%	QNQQEKNE
79.4 ^{a)}		1.6x10 ³	1:16	85%	EKNEQEELLE
71.1		4x10 ²	0	0%	QQEKNEQE
71.2		6.4x10 ³	1:16	95%	QELLELDKW
71.3		1.6x10 ³	1:8	70%	QQEKNEQE
91.1		1.6x10 ³	0	0%	ELDKWASLW
91.2		1.6x10 ³	≥ 1:4	90%	DKWASLWNFN
91.3		1.6x10 ³	0	0%	NEQEELLE
80.1		0.1mg p15E/gp41 hybrid I, E2-peptide	1.6x10 ³	0	0%
80.2	1.6x10 ³		0	0%	EKNEQEELLE
80.3	1.6x10 ³		0	0%	EKNEQEELLE
80.4	1.6x10 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 hybridoma 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: EKNEQEELLELDKWASLWNWFNITNWL

^{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.

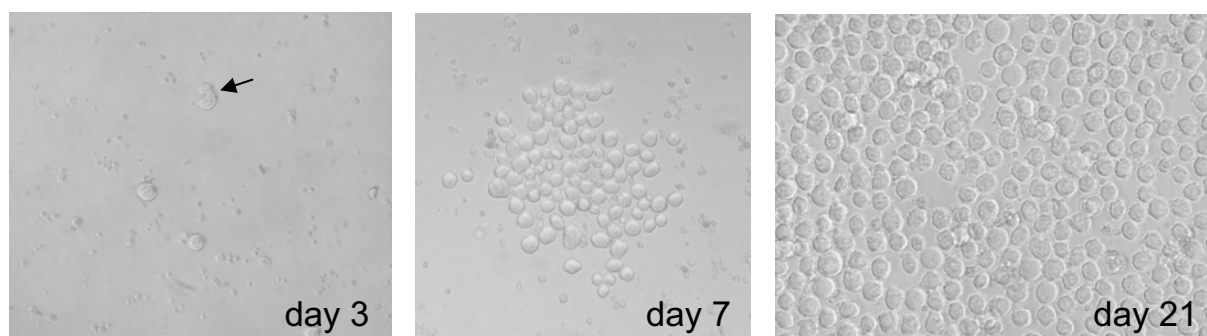


Figure 36 Hybridoma cell cultures at days 3, 7 and 21 after fusion at a magnification of 200x.

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).

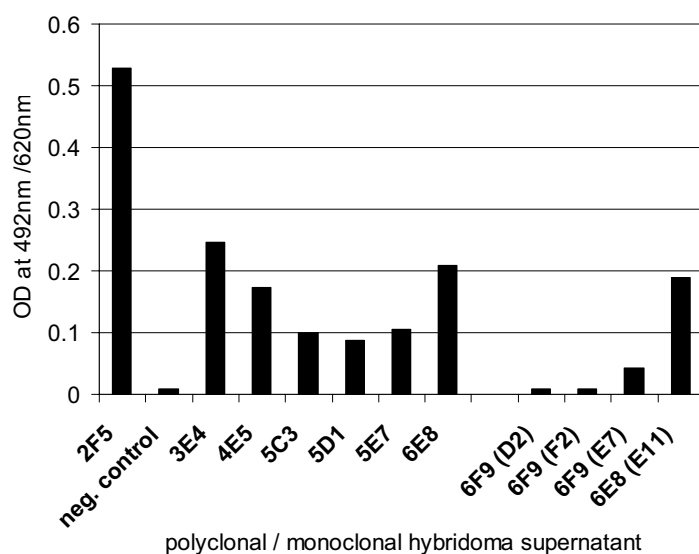


Figure 37 Example of an ELISA screening for hybridoma cell culture supernatants after first and second singling of clones. Clones with denotations in brackets were singularised twice and supposed to be monoclonal.

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).

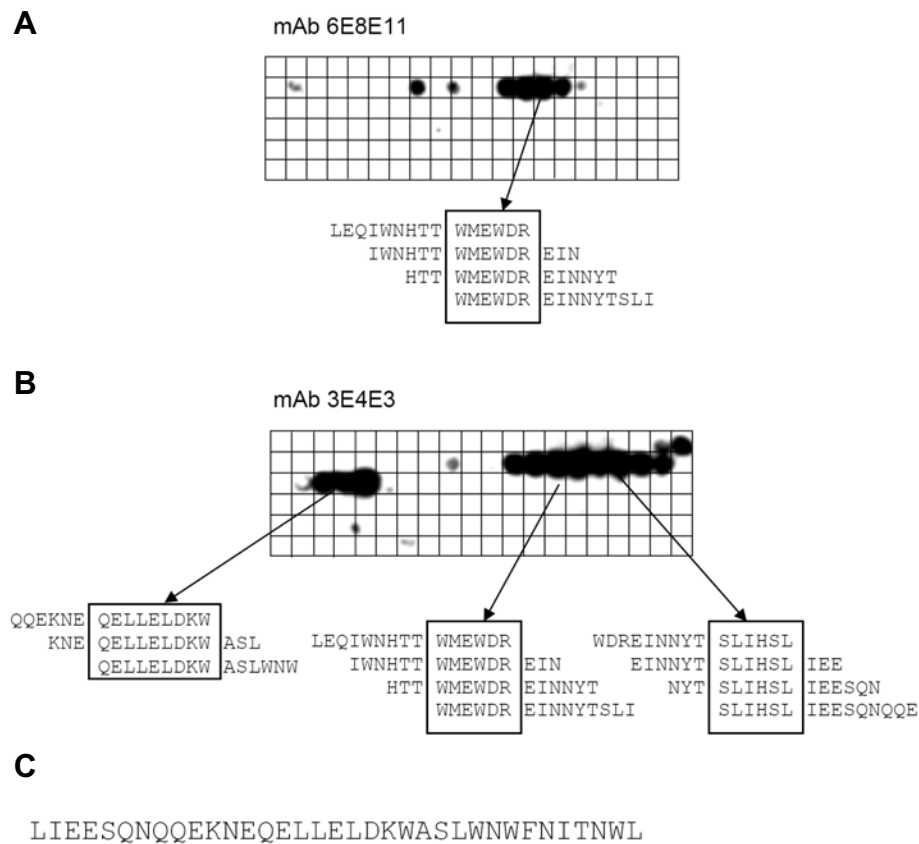


Figure 38 Epitope mapping using peptid spot membranes with overlapping peptides and hybridoma cell culture supernatants. **(A)** Results obtained with the mAb 6E8E11 and **(B)** results obtained with the mAb 3E4E3. The sequences of the reacting peptides are shown and the identified epitopes are framed. **(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.

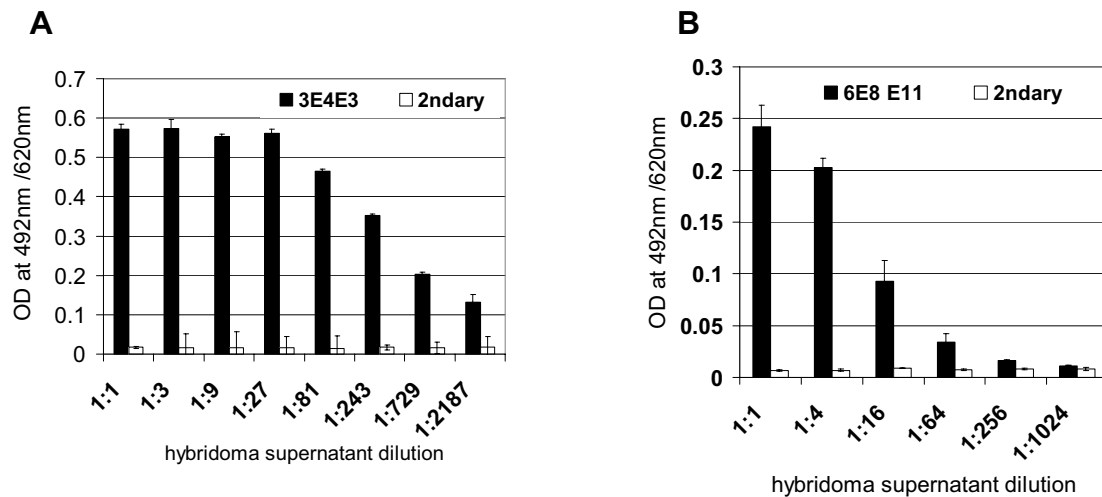


Figure 39 Reactivity of mAb3E4E3 in a three fold serial dilution (**A**) and of mAb6E8E11 in a four fold serial dilution (**B**) with the HIV-1 gp41 E2 peptide.