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Short communication

Immunization with the transmembrane protein of a retrovirus, feline leukemia virus: Absence of antigenemia following challenge

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ABSTRACT

A major challenge in the development of vaccines against retroviruses is the induction of neutralizing antibodies since they prevent infection of the cells where the virus may persist. The transmembrane envelope (TM) protein contains highly conserved domains and seems to be a suitable target. To study whether vaccinating with a TM protein of a retrovirus could protect from infection *in vivo*, cats were immunized with the TM protein p15E of feline leukemia virus (FeLV) and subsequently challenged. For the first time we show that immunization with a retroviral TM protein prevented antigenemia. The level of neutralizing antibodies after the boost immunization correlated with the outcome of FeLV infection.

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Vaccines protecting cats from FeLV-induced diseases are well described, however their mechanism of protection is unclear. Either antibodies (Hoover et al., 1977; Jarrett et al., 1977) or cytotoxic T cells (Flynn et al., 2000) are thought to be protective. Retroviruses integrate as proviruses into the genome of the infected cell. The induction of neutralizing antibodies is of great advantage since they prevent infection before integration and persistence. Surface (SU) and transmembrane envelope (TM) proteins are the natural targets of neutralizing antibodies.

Due to important functions in the infection process, the TM proteins of all retroviruses are highly conserved (Weiss, 2003). This is especially evident in the case of HIV-1: the SU protein gp120 is highly variable, whereas the TM protein gp41 is conserved and target of broadly neutralizing antibodies isolated from HIV-1 infected individuals (Muster et al., 1993; Zwick et al., 2001). In contrast to unsuccessful attempts to induce such antibodies against HIV-1, we previously succeeded in raising neutralizing antibodies against porcine endogenous retrovirus (PERV) (Fiebig et al., 2003), feline leukemia virus (FeLV) (Langhammer et al., 2005, 2006, 2010) and Koala retrovirus (Fiebig et al., 2006) by immunizing with their corresponding p15E TM protein. Despite the well known immunosuppressive properties of retroviral TM proteins (Denner, 2010) immunizations induced binding and neutralizing antibodies. In order to study whether neutralizing antibodies specific for the TM protein can protect cats from infection *in vivo*, they were immunized with p15E of FeLV and subsequently challenged.

Animals (6–26-month-old outbred cats with equal distribution of weight and age) were obtained from the Animal Facility of the University Düsseldorf and kept under identical conditions. Six animals were immunized twice intramuscularly with 500 μg purified FeLV-A p15E in Montanide ISA 720, an experimental squalene adjuvant that contains a mannide monooleate emulsifier (Langhammer et al., 2006) (Fig. 1). Three cats (numbers 1.1–1.3) served as non-immunized control. The cats were challenged oronasally with daily 1 ml doses of 1×10^6 ffu FeLV-A Glasgow-1 strain (Harbour et al., 2002) on four consecutive days. Blood was taken 3 days before challenge and 10, 30, 60, 80, 100 and 150 days after challenge.

Since in vaccine trials, protection from FeLV challenge is conventionally indicated by a failure of the establishment of a persistent viremia or antigenemia, measured by the absence of either infectious virus or FeLV p27Gag antigen in the blood, sera were tested for p27 Gag using a commercial detection assay (Feline leukemia virus antigen test kit, Synbiotics). Levels of p27Gag were expressed as percentage setting the level of p27Gag in a serum from a highly viraemic FeLV positive cat as 100% (Fig. 2, p27).

None of the cats showed p27Gag in the blood when tested 3 days before and 10 days after challenge. 30 days after the challenge, p27Gag was detected in the sera of all non-immunized control cats (Fig. 2A, animals 1.1, 1.2, 1.3) considering them FeLV positive. Analyzing the animals immunized with p15E, cats 2.1 and 2.5 were p27Gag positive and all others were negative 30 days after challenge. At day 60 cat 2.6 also became positive, whereas cats 2.2–2.4 remained p27Gag negative until the end of the study. Therefore 50% of cats immunized with p15E were considered to be protected from antigenemia.

The neutralizing capacity of the cat sera was determined in a neutralization assay infecting feline embryonic fibroblasts FEA with

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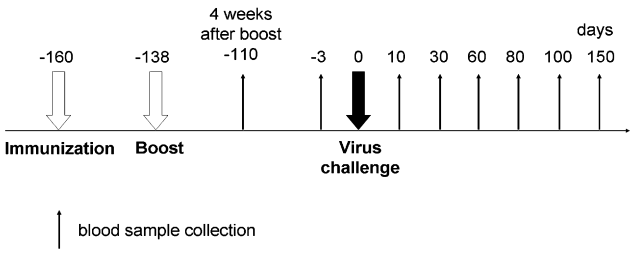


Fig. 1. Time schedule of the immunization-challenge experiment. Cats were immunized at day 160 and boosted at day 138 before challenge. Parameters such as binding antibodies, neutralizing antibodies, p27Gag and provirus load in blood were investigated 110 and 3 days before and 10, 30, 60, 100 and 150 days after virus challenge.

FeLV-A and measuring decrease of provirus integration by virus specific real-time PCR based on the comparative ct method (for details see Langhammer et al., 2005). The neutralizing activity was expressed as percent neutralization compared to the corresponding preimmune serum at serum dilution of 1:100. Four weeks after the boost immunization (day −110), all sera showed neutralizing activity. Animals 2.2, 2.3 and 2.4 showed high neutralizing activity (all above 78%), while animals 2.1, 2.5 and 2.6 had low or medium activity (all below 60%) at day −110. Sera from all animals protected from antigenemia (cats 2.2, 2.3 and 2.4) had the highest neutralizing activity four weeks after the boost immunization (day −110) but not 3 days before and at any time point after challenge (Fig. 2B). This result suggests that measuring neutralizing activity at four weeks after boost can give an indication of possible resistance to infection.

The neutralizing activity in the sera from animals immunized with p15E increased during the course of infection. Sera from animals 2.2, 2.3 and 2.4 protected from antigenemia showed a neutralizing activity of 100% at day 150. In contrast, animals 2.1, 2.5 and 2.6, with detectable p27Gag levels in blood, never reached neutralizing activities above 70% (Fig. 2B). Challenge with infectious virus also induced neutralizing antibodies in non-immunized animals. The serum from non-immunized animal 1.3 showed high neutralizing activity at days 10, 30 and 150 (up to 90%) and was p27Gag negative at day 150 (Fig. 2A). In contrast the sera

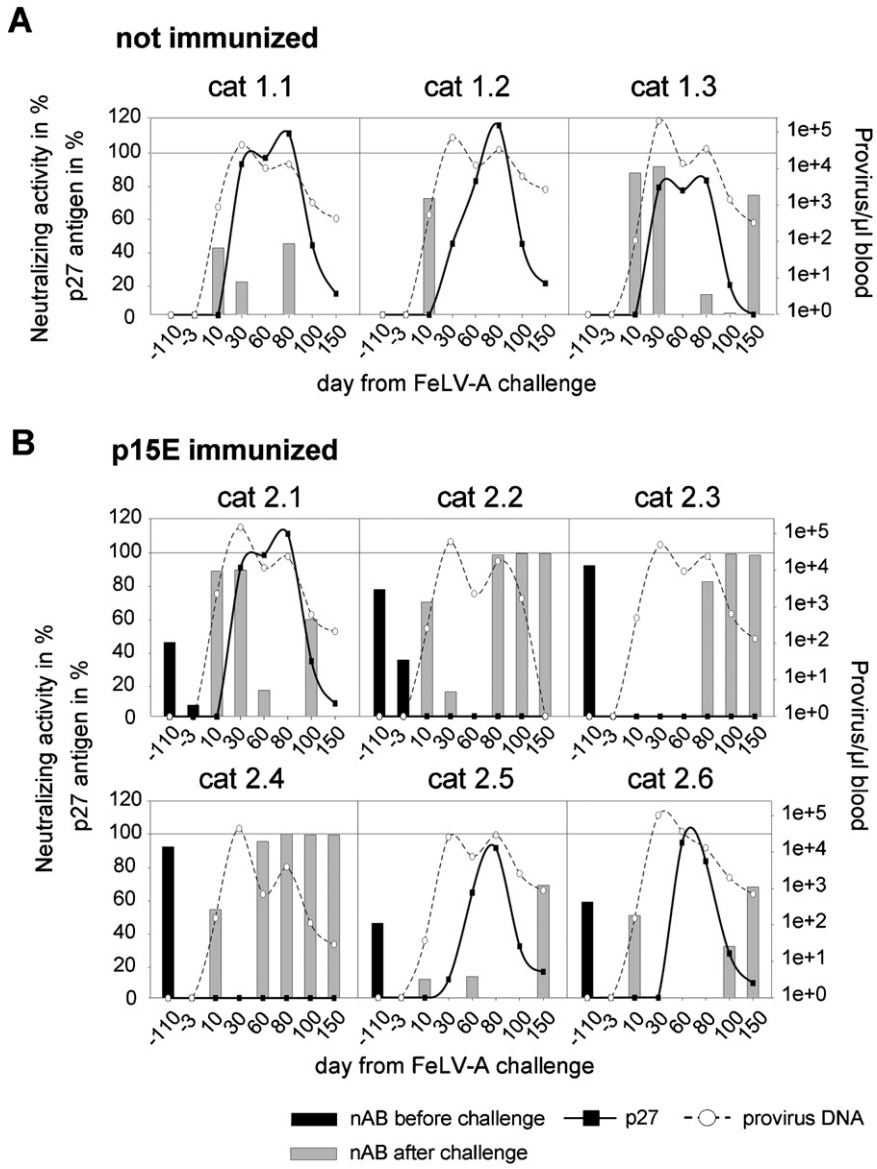
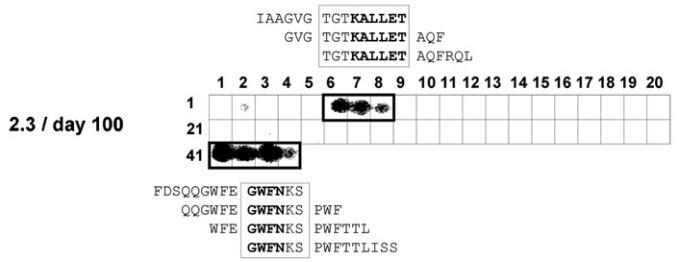


Fig. 2. FeLV p27 antigen, provirus DNA and neutralizing activity in non-immunized animals (A) and in animals immunized with p15E (B) before and after challenge with FeLV-A. p27 antigen values is expressed as percentage (black line, scale indicated at left *y*-axis). FeLV neutralizing activity before/after virus challenge is presented in black/grey bars (scale indicated at the left *y*-axis). FeLV-A provirus integration is shown in copies/µl peripheral blood (dotted line, scale indicated at the right *y*-axis).

A



B

Epitope	Sequence	2.1	2.2	2.3	2.4	2.5	2.6
		100	100	100	100	100	100
E1a	**KALLETAQF**RQL	+	+	+	+	+	+
E1b	**ALEESISALEKS**	+	-	-	-	+	-
E2a	VRD**MAKLRERLKQRQQL**	+	-	-	+	+	+
E2b	**WFEGWFN**KSP	-	+	+	+	-	-

-: no detection; +: detection; grey background: absence of antigenemia

C

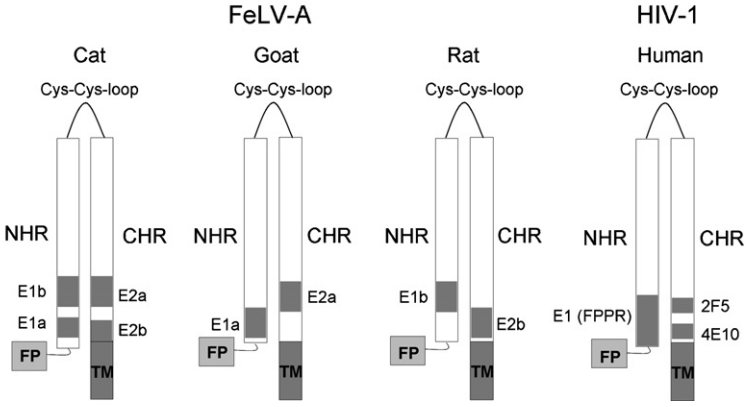


Fig. 3. Epitope mapping of cats immunized with p15E and challenged with FeLV-A. (A) As an example, mapping of cat serum 2.3 is shown, identified epitopes were framed and core epitope sequences are printed in bold. (B) Results of the epitope mapping using sera from cats immunized with p15E and challenged with FeLV at day 100. Core epitope sequences are printed in bold. (C) Schematic presentation of the localization of the epitopes recognized by neutralizing sera from goats, rats (Langhammer et al., 2005) and cats (Langhammer et al., 2006) immunized with p15E of FeLV in comparison with the localization of the epitopes of antibodies 2F5 and 4E10 broadly neutralizing HIV-1 in gpp41 of HIV-1. FP–fusion peptide, NHR–N-terminal helical region, CHR–C-terminal helical region, TM–transmembrane region, E1a, E1b, E2a, E2b, 2F5, 4E10 indicate the epitopes, E1 (fusion peptide proximal region, FPPR) indicates the peptide in the fusion peptide proximal region of gp41 enhancing binding of 2F5 and 4E10 to their epitopes (Fiebig et al., 2009).

from the other non-immunized animals only showed low levels of neutralizing antibodies and were p27Gag positive at all time points.

To measure provirus load, DNA from 100 μl blood was purified using a whole blood kit (Qiagen) and assessed by specific real time PCR (Langhammer et al., 2005). Provirus load was calculated as FeLV-A genome copies/μl blood (Fig. 2). In none of the samples taken before challenge provirus was detected, confirming that uninfected animals were used for the study.

All cats became provirus positive after challenge, demonstrating that immunization with p15E could not prevent infection. Commercial FeLV vaccines such as Eurifel, a canarypox-vector-based live vaccine expressing Env, Gag and Pol, of FeLV-A as well as Fel-O-Vax, an inactivated whole virus vaccine also failed to induce sterilizing immunity although they protect in all cases from disease development (Hofmann-Lehmann et al., 2005). However, sterilizing immunity in a retroviral system is possible, since adoptive transfer studies using broadly neutralizing antibodies including 2F5 and 4E10 directed against the TM protein gp41 of HIV-1 prevented provirus integration (Ruprecht et al., 2003). Better immunization strategies, including combination of different viral targets, higher doses of antigen and a better presentation of the TM protein may increase the neutralizing activity of the induced antisera.

Provirus load was similar in non-immunized and immunized animals. Provirus integration was detected starting with day 10 after challenge (108 and 126 copies/μl blood). At day 30 a peak with up to 5.82×10^4 and 2.66×10^5 copies/μl blood was reached, which decreased with time (Fig. 2). Astonishingly, in cats 2.2, 2.3 and 2.4 with no p27Gag antigenemia similar provirus DNA levels were found. The lowest proviral levels were observed in cats 2.2 (negative at day 150) and 2.4 (≤120 copies/μl blood starting with day 100). At the same time (day 100 and day 150) a high provirus load was detected in cats 2.5 (2890 and 976 copies/μl blood) and 2.6 (2202 and 769 copies/μl blood) (Fig. 2B). These data correlate with recent findings that no difference was found in initial provirus load between cats with different infection outcome (Hofmann-Lehmann et al., 2008).

A late analysis of immunized and non-immunized animals at day 960 supports the partial efficacy of immunization with p15E (Table 1). At that time point two out of three control animals (1.1 and 1.3) and one p15E immunized but unprotected animal (2.1) had died. Most importantly only the three animals immunized with

Table 1
Long term examination of non-immunized cats and cats immunized with p15E at day 960 after virus challenge. Animals 2.2, 2.3, 2.4 marked grey had been found protected from antigenemia after challenge.

Antigen	Cat	Status	p27 antigen	Provirus
None	1.1	[a](908)	–[b]	+[b]
	1.2	[a](959)	+[b]	+[b]
	1.3	Alive	–	+
p15E	2.1	[a](895)	+[b]	+[b]
	2.2	Alive	–	–
	2.3	Alive	–	–
	2.4	Alive	–	–
	2.5	Alive	+	+
	2.6	Alive	+	+

[a] Animal deceased at indicated day.
[b] Results obtained from analysis immediately after decease.

p15E and not showing antigenemia were negative for provirus at day 960 (cats 2.2, 2.3 and 2.4). This late result supports the findings made at day 150 after virus challenge.

In order to analyze the epitopes recognized by the immune sera, an epitope mapping was performed using overlapping peptides corresponding to the entire p15E of FeLV Glasgow-1 strain at dilutions of 1:1000 (Fig. 3A). When the epitope mapping was performed with antisera collected 100 days after virus challenge, only sera from aviremic cats, but not sera from the viremic animals, recognized the E2b epitope WFEGWFNKSP (Fig. 3B). In addition, four epitopes, designated E1a, E1b (located in the fusion peptide proximal region, FPPR), E2a, and E2b (located in the MPER) were described, confirming previous results of analysis immune sera from rats, a goat and cats which had been immunized with the TM protein (Fig. 3C) (Langhammer et al., 2005, 2006). Despite the evolutionary distance between HIV and gammaretroviruses there is a similar location in the MPER and a sequence homology between the epitope of 4E10 (NWFNIT) and E2b (WFEGWFNKSP, identical amino acids underlined) was observed. A second antibody also broadly neutralizing HIV-1, 2F5, and isolated from a HIV-infected individual, recognizes another epitope in the MPER of gp41 (Muster et al., 1993; Zwick et al., 2001) (Fig. 3C). However, until now the induction of such antibodies by active immunization with a variety of antigen constructs in different animal models including non-human primates has not been successful (Ho et al., 2005; Joyce et al., 2002; Lu et al., 2000). Interestingly, a peptide corresponding to the FPPR (E1) was shown to increase the binding of the antibodies 2F5 and 4E10 to a peptide containing their epitopes (Fiebig et al., 2009).

The epitope mapping data of the cat sera is in good agreement with the results after immunization of goats and rats with p15E of PERV (Fiebig et al., 2003) and KoRV (unpublished data) confirming the two prominent epitope regions, one in the FPPR (E1) and one in the MPER (E2).

The observations in this study might have implications for the development of antiretroviral vaccines advising to include their highly conserved TM proteins to provide protection. The high conservation of the sequence based on important functions during infection might prevent escape from immune control. It is well known that retroviral TM proteins are immunosuppressive (for review see Denner, 1998, 2010). In contrast to the situation during infection, which is characterized by a permanent high viral load and a high load of the immunosuppressive TM protein, its concentration during vaccination is low but still allowing an effective immune response. Since there is evidence that mutation of the immunosuppressive domain of the TM proteins results in a higher immune response (Schlecht-Louf et al., 2010, and our unpublished data), the use of mutated TM proteins should be evaluated in well-designed studies involving a large number of target animals.

Conflict of interest

The authors declare no conflict of interest.

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