Increased Neutralizing Antibody Response after Simultaneous Immunization with Leucogen and the Feline Leukemia Virus Transmembrane Protein

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Key Words

Feline leukemia virus • Vaccination • Neutralizing antibodies • Transmembrane envelope protein

Abstract

To develop improved vaccination strategies against feline leukemia virus (FeLV), rats were immunized with the transmembrane envelope protein p15E of FeLV alone or in combination with the commercial vaccine Leucogen® comprising the nonglycosylated FeLV surface envelope protein. Binding and neutralizing antibodies were induced in both groups and in the group immunized with Leucogen alone. Higher titers of antibodies neutralizing FeLV were induced by simultaneous immunization with Leucogen and p15E compared to the responses using Leucogen or p15E alone, suggesting that combination vaccines should be used in the future. Epitope mapping of p15E-specific antibodies induced by simultaneous immunization with Leucogen and p15E revealed the same pattern of response as obtained after immunization with p15E alone: one epitope was localized in the membrane-proximal external region (MPER) and the other in the fusion peptide-proximal region, and they are related to the epitopes detected after immunization with p15E of the porcine endogenous retrovirus and the koala retrovirus. The data indicate that these epitopes in the MPER are an effective target for neutralization and that antigens containing them may therefore prove to be a useful component of vaccines against retroviruses, including HIV-1.

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Introduction

Immunization is the most effective method to prevent diseases caused by infectious agents. The success of a variety of vaccines against diseases such as smallpox, measles, mumps, and poliomyelitis has dramatically reduced morbidity and mortality worldwide and has led to the eradication of smallpox [1]. It is of interest that most, if not all, successful vaccines are based on the induction of neutralizing antibodies. Although the development of vaccines against human immunodeficiency virus-1 (HIV-1), the retrovirus that causes AIDS, is one of the major tasks of present vaccine development, all attempts up to now have failed [2]. In addition, passive immunization using broadly neutralizing monoclonal antibodies such as 2F5 and 4E10 derived from HIV-infected patients has been used to treat HIV infection [3]. The epitopes recognized by monoclonal antibodies 2F5 and 4E10 are located in the membrane-proximal external region (MPER) of gp41 [4, 5]. Numerous attempts to induce broadly neutralizing antibodies such as 2F5 and 4E10 using recombinant gp41 or synthetic peptides have failed [6], although, recently, oral immunization with bovine papilloma virus/HIV-1 gp41 chimeric virus-like particles induced very low titers of neutralizing antibodies in mice [7].

In contrast, vaccines against feline leukemia virus (FeLV) are commercially available, showing that antiretroviral vaccines are not impossible. FeLV is a gammaretrovirus comprising 3 subtypes, i.e. A, B and C [8], of

which FeLV-A is the predominant serotype in cats [9]. FeLV induces fatal leukemia, lymphomas, and immunosuppression associated with opportunistic infections in infected cats. 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 7 commercial FeLV vaccines currently available in the USA and Europe provide 100% protection against infection. Three vaccines are composed of inactivated whole virus, 2 are gp70 subunit vaccines, and 2 are recombinant vaccines [10]. The Leucogen[®] vaccine contains the recombinant nonglycosylated p45 of the surface envelope glycoprotein gp70 and is one of the most effective [11].

Recently, we induced neutralizing antibodies specific for the transmembrane envelope protein p15E of porcine endogenous retrovirus (PERV) [12], FeLV-A [13, 14], and koala retrovirus (KoRV) [15] in goats, rats, and cats. The epitopes recognized by the immune sera were located in 2 distinct regions of the transmembrane envelope protein including the MPER, and a sequence homology between the epitopes in the MPER of FeLV, KoRV, PERV, and HIV-1 was reported [12]. Our data suggest that these regions of the transmembrane envelope protein represent a vulnerable target for neutralization. Therefore, this antigen may prove to be a useful component of all antiretroviral vaccines.

Here we describe for the first time the induction of higher titers of neutralizing antibodies in rats by combined immunization with the surface envelope protein in the form of the commercially available Leucogen vaccine and the recombinant ectodomain of p15E of FeLV, and we describe the localization of the epitopes recognized. We show that a vaccine containing both antigens is more powerful compared to each single antigen. These findings are of great importance for the general improvement of retroviral vaccines.

Materials and Methods

Experimental Animals, Antigens, and Immunization

Eighteen Wistar rats (Charles River, Wilmington, Mass., USA) were immunized twice intramuscularly and subcutaneously at weeks 0 and 3. Leucogen, containing 0.1 mg p45 plus Quil-A and aluminium hydroxide as adjuvants (Virbac; lot No. 80986902143521), was given alone or mixed with 100 µg p15E. Immunization with 100 or 500 µg p15E alone was performed at a dilution of 7:3 in Montanide ISA 720 (Seppic, France; lot No. 143521). The recombinant ectodomain of p15E of FeLV-A used for immunization was prepared as described [13]. Briefly, the ectodomain (aa 476–583) of FeLV-A p15E was cloned into the pCal-n

vector (Stratagene Europe, Amsterdam, The Netherlands) and expressed in *Esterichia coli* BL21 DE3 cells. The p15E N-terminally fused to a 4-kDa calmodulin-binding protein was purified by affinity chromatography.

Quantification of Antigen-Specific Immunoglobulins by ELISA

FeLV-A p15E and Leucogen p45-specific antibody titers were determined by ELISA. Briefly, ELISA plates were coated for 1 h at 37° with affinity-purified recombinant p15E protein diluted in PBS or for 18 h at 37° with Leucogen p45 vaccine component diluted in PBS (100 ng/well). The ELISA plates were then washed 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 with PBS containing 2.5% BSA and 0.1% Tween 20, were added to the ELISA plates at a starting dilution of 1:1,000, diluted further (4-fold dilution series), and incubated for 1 h at 37°. The ELISA plates were then washed 3 times with PBS containing 0.1% Tween 20. A horseradish peroxidase-conjugated secondary antibody specific for rat IgG diluted 1:3,500 with PBS containing 2.5% BSA and 0.1% Tween 20 was used to detect antigen-specific immunoglobulin. Incubation for 1 h at 37° was followed by 5 washings with PBS containing 0.1% Tween 20. Finally, the ELISA plates were developed by addition of OPD (*o*-phenylenediamine dihydrochloride) diluted in PBS (50 µg/well) plus 0.1% H₂O₂ followed by inactivation with 30 µl H₂SO₄ (5 N) after 10 min. Protein-specific antibody endpoint titers are reported as the dilution giving an OD_{492/620 nm} reading above that of preimmune sera.

Virus Neutralization Assay

The virus stock for the neutralization assay was prepared as cell-free supernatant from feline embryonic fibroblast (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 was shown to have a titer of 10^{4.76} TCID₅₀/ml. Neutralization assays were performed as follows: 1 day before the assay, 6,000 uninfected FEA cells per well were seeded into 96-well microtiter plates. Preimmune and immune sera were heat-inactivated at 56° for 30 min. 50 µl of stock virus were incubated with 4-fold serial dilutions of serum for 30 min at 37° and then transferred to the cells. After 3 days of incubation, cells were freeze-thawed 3 times and a lysis buffer containing 20 mg/ml of proteinase K in PCR buffer (50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl; pH 8.4) was added. The cells were incubated for 3 h at 56° followed by 10 min at 95° to inhibit proteinase K activity. Proviral DNA was measured by real-time PCR as described below. To determine the titer of neutralizing antibodies, the dilution with provirus integration less than 100% was determined.

Real-Time PCR

An internal probe FAM-5'-TTAAGCACCTGGGCCCCGGC-3'-DQ (Eurogentec, Cologne, Germany) was used together with FeLV-specific primers. The sense primer 5'-TCAAGTATGTTC-CCATGAGATACAA-3' and the antisense primer 5'-GAAGGTCGAAGTCTGGTCAACT-3' were used to amplify and to quantify a 185-bp product from the exogenous U3 sequence in the LTR region of the FeLV-A provirus genome. The 25-µl reaction mixture consisted of 1 × PCR buffer with 1 mM MgCl₂; 0.5 µM each

of dATP, dCTP, dGTP, and dTTP; 5 pmol of each primer; 5 pmol of probe; 1.25 U Amplitaq Gold polymerase, and 2 μ l lysis mixture. The thermal cycling conditions used were 12 min at 95° followed by 50 cycles of 1 min at 95°, 1 min at 59° and 30 s at 72° in a Stratagene MX4000 system.

Epitope Mapping

The entire p15E of the FeLV-A Glasgow strain was synthesized as a cellulose-adsorbed peptide spot library of 15-mer peptides overlapping by 13 amino acids (Jerini Biotoools, Berlin, Germany) using a standard protocol. Sera were diluted 1:1,000 and incubated with the membrane for 3 h, washed 3 times for 15 min with Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 (Sigma-Aldrich, Steinheim, Germany) and incubated for 2 h with a peroxidase-conjugated secondary antibody diluted 1:10,000. Binding was detected using a chemiluminescence detection solution (ECL, Amersham Pharmacia Biotech).

Results

The Transmembrane Envelope Protein p15E of Different FeLV Is Highly Conserved

The transmembrane envelope proteins of retroviruses are very similar in their structure due to their specific functions during infection. They are associated with conformational changes including an intramolecular interaction between the N-terminal helix and the C-terminal helix [16]. Although the surface envelope protein gp70 of gammaretroviruses is 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. To show this, the amino acid sequences of gp70 and p15E from the FeLV-A strains Glasgow-1, Rickard, and Sarma and the FeLV-B strain Gardner-Arnstein were aligned and analyzed as a phylogenetic tree using the program MegAlign (part of the DNASTAR package). A comparison of the phylogenetic trees for p15E and gp70 showed a higher divergence for the gp70 proteins than for the p15E proteins (fig. 1a). After having immunized rats, goats, and cats with the transmembrane envelope protein p15E of FeLV [18, 19], binding and neutralizing antibodies were induced and epitopes designated E1a, E2a, E1b, and E2a were identified in the fusion peptide-proximal region (FPPR) and membrane-proximal external region (MPER) of p15E (fig. 1b). The amino acid sequences of these epitopes are 100% identical in the strains analyzed (fig. 1c). These data indicate that the transmembrane envelope protein, especially the MPER, is a highly conserved target for neutralization and may be used to induce antibodies neutralizing all FeLV strains.

Neutralizing Activity Correlated with the Recognition of Epitope E1b

In order to induce neutralizing antibodies, a group of 6 rats (animals 1.1–1.6) was immunized with the ectodomain of the transmembrane envelope protein p15E of FeLV-A. Two out of 6 antisera obtained after boost immunization were able to inhibit the infection of FEA cells by FeLV-A. Both antisera showed neutralization titers of 1:4 as determined by a 4-fold serum dilution. The percentages of provirus integration for antisera 1.2 and 1.4 were reduced to 25 and 4%, and the neutralizing capacities were 75 and 96%, respectively (fig. 2a). All antisera from this animal group showed strong binding antibodies against the recombinant p15E protein in ELISA (fig. 2b). Their titers of binding antibodies were in a range between 2.56×10^5 and 4.1×10^7 ; however, they did not correlate with the neutralizing capacity of the sera. When epitope mapping was performed using linear 15-mer peptides (overlapping by 13 amino acids) corresponding to the entire p15E of FeLV-A, covalently bound by the C-terminus to a cellulose membrane, 2 main epitopes were identified, i.e. E1b and E2b (fig. 2c, d). This finding confirms previous results showing the induction of neutralizing antibodies and the identification of the same epitopes after the immunization of goats and rats with FeLV-A p15E [13]. Interestingly, all antisera immunized with FeLV-A p15E recognized the E2b epitope but only antisera with neutralizing activity also recognized the E1b epitope (fig. 2c, d; antisera 1.2 and 1.4).

Immunization with Leucogen Induced Binding and Neutralizing Antibodies against p45 in Rats

To analyze whether Leucogen, which is comprised of the recombinant unglycosylated envelope surface protein p45 of FeLV, induces, as expected, binding and neutralizing antibodies a group of 3 rats was immunized with 1 dose and boosted with a second dose of Leucogen. All 3 antisera obtained showed neutralization titers of 1:16. The neutralization capacity ranged from 80 to 100% at a serum dilution of 1:4 (fig. 3a). These data showed that the induced neutralizing capacity in vitro is much higher after immunization with Leucogen when compared to immunization with p15E (fig. 2a). Binding antibodies from all 3 sera showed titers of 6.4×10^4 against recombinant p45 as determined by ELISA (fig. 3b). Therefore, the titers of binding antibodies induced by immunization with p15E (fig. 2b) were significantly lower when compared with the titers against p45 obtained from immunization with Leucogen (fig 2a).

Fig. 1. a Comparison of p15E of different FeLV; unrooted phylogenetic tree of gp70 and of p15E of the FeLV strains Glasgow-1, Rickard, Sarma (FeLV-A), and Gardner-Arnstein (FeLV-B). **b** Schematic presentation of the transmembrane envelope protein p15E of FeLV. NHR = N-Terminal helical region; CHR = C-terminal helical region. C-C cysteine loop, E1a, E1b, E2a, and E2b indi-

cate the epitopes recognized by antisera induced by immunization with the ectodomain of p15E [13]. **c** Alignment of epitopes designated E1a, E2a, E1b, and E2a identified after the immunization of rats with the recombinant ectodomain of p15E of FeLV [13] (framed and indicated in grey) in different FeLV-A strains.

Fig. 3. Results of the immunization of rats with Leucogen. **a** Neutralizing activity. **b** ELISA reactivity against p45.

Combined Immunization with p15E and Leucogen Is Superior to Immunization with Leucogen Alone

When the neutralizing capacity of the sera obtained after combined immunization with Leucogen and p15E (group 3) was compared with that of the sera obtained after immunization with p15E (group 1) or Leucogen alone (group 2), higher neutralization titers were observed in sera from group 3 (fig. 4a). All sera from rats immunized with the combination of Leucogen and p15E showed neutralizing activity against FeLV-A. Eight of 9 sera showed a neutralization titer $\geq 1:64$, whereas only 1 serum (3.7) had a lower titer of 1:16 (fig. 4a). Five of 9 sera showed binding antibody titers against p15E comparable to those obtained from immunizations with p15E alone (fig. 4b). The other 4 antisera showed lower antibody titers of 6.4×10^4 (fig. 4b; sera 3.5, 3.7, 3.8, and 3.9). A similar observation was made for antibody titers against p45: 5 out of 9 sera had titers comparable to those obtained from immunizations with Leucogen alone, but the other 4 showed lower titers of 1.6×10^4 (fig. 4b; antisera 3.4, 3.5, 3.7, and 3.8). These observations indicated that

combined immunizations with p15E and Leucogen lead to reduced titers of binding antibodies on the one hand, but to an increased efficacy of neutralizing antibodies against FeLV on the other.

Epitope mapping of the sera from animals immunized with a combination of p15E and Leucogen detected all 4 epitopes (E1a, E1b, E2a, and E2b) as described previously [13]. The only epitope that all the antisera of this group recognized in common was the E1b epitope (fig. 4c). This epitope was also detected by both neutralizing sera obtained from immunizations with p15E alone (fig. 2d). Similar to the group immunized with p15E alone, nearly all sera obtained after combined immunizations of p15E with Leucogen recognized the E2b epitope (fig. 4c). Only the serum from animal 3.7 failed to detect the E2b epitope. Interestingly, this antiserum not only showed the lowest titer of neutralizing antibodies (fig. 4a) but also reduced titers of binding antibodies against both p15E and p45 (fig. 4b). Taken together, this data indicated that recognition of E1b and E2b seems to be associated with neutralizing activity.

Fig. 4. Results of the combined immunization with p15E and Leucogen. **a** Neutralizing activity. **b** ELISA reactivity against p45 and p15E. **c** Schematic presentation of the epitopes of all immune sera obtained by epitope mapping. The titers of neutralizing antibodies are indicated on the right.

Discussion

Immunizing rats with the transmembrane envelope protein p15E of FeLV induced neutralizing antibodies which react with 2 epitopes: 1 located in the FPPR and the other in the MPER. This confirms previous results showing that similar neutralizing antibodies were also obtained in goats and rats [13]. Most importantly, higher titers of neutralizing antibodies were obtained when a combined immunization with p15E and a licensed vaccine, i.e. Leucogen, was performed, indicating that this vaccine can be improved.

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 nonglycosylated surface envelope protein p45. For all commercially available FeLV vaccines, there is only limited scientific data concerning the

long-term duration of immunity after vaccination and none of the studies demonstrate 100% protection [17, 18]. Therefore, annual revaccination is recommended. None of the vaccines regularly showed neutralizing antibodies; such antibodies are usually detected only after recovery from challenge [17]. However, a high proportion of cats are protected by vaccination, suggesting that immune mechanisms other than neutralizing antibodies may be involved. This conclusion was strengthened 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 [19, 20]. However, it should not be concluded from these findings that neutralizing antibodies are not important. The presence of neutralizing antibodies in cats recovering from natural FeLV infection clearly correlates with a resistance to infection and the passive transfer of antibodies, either naturally through the colostrum [21] or experimentally by infusion [22], protecting cats against FeLV challenge.

Data presented here clearly show that immunization with Leucogen alone (group 2) was able to induce neutralizing antibodies (fig. 3a). This also indicates that our neutralization assay based on the measurement of provirus integration by real-time PCR is highly sensitive to such antibodies.

Numerous attempts have been undertaken to improve the efficacy of vaccination. For example, immunization with immunostimulating complexes (ISCOM) containing the gp70/gp85 precursor molecules not only induced virus neutralizing antibodies, but also protection against infection [23]. The development of antibodies to gp70 and p15E was confirmed by Western blot [24]. When compared with the commercial vaccine Leucocell, containing inactivated FeLV, the ISCOM preparation proved to be superior. Virus-neutralizing antibodies were also induced by synthetic peptides corresponding to a domain of gp70 of FeLV-A involved in infection [25]. Since FeLV-B and FeLV-C might originate from a recombination between FeLV-A and endogenous FeLV-related sequences [26], neutralizing antibodies against FeLV-A should also protect cats from natural infection with all subgroups. The use of the transmembrane envelope protein described here is a new approach to induce broadly neutralizing antibodies which has been shown to be successful in the case of PERV [12], KoRV [15], and FeLV in different animals including cats [13, 14].

In previous immunization experiments with p15E of FeLV-A, the induced antisera neutralized FeLV-A and 2–4 epitopes were identified in the ectodomain of FeLV-A

p15E [13, 14]. These same epitopes were recognized when p15E was used for immunization together with Leucogen (fig. 4c). Two epitopes, designated E1a (LETAQFRQL) and E1b (IQALEESISALEK), were localized in the FPPR; the other 2, E2a (KQRQQL) and E2b (WFEGWFN) (amino acids identical with an epitope recognized by antibodies broadly neutralizing HIV-1 are in bold), were localized in the MPER. It is important to underline that epitopes E2a and E2b correspond in their localization to the epitopes of 2F5 and 4E10, 2 monoclonal antibodies generated from HIV-1-infected individuals, broadly neutralizing HIV-1 and that there is – despite the evolutionary distance between HIV-1 and gammaretroviruses such as FeLV, KoRV, and PERV – a sequence homology of at least 3 amino acids. The epitope of the antibody 4E10 broadly neutralizing HIV-1 is **NWFNIT** (identical amino acids in bold) [5]. There is evidence that recognition of the epitopes E1b and E2b is crucial for neutralization (fig. 2) and that simultaneous immunization with p15E and Leucogen changed the pattern of recognition by increasing the number of sera binding to E1a and E1b. It may be speculated that during immunization an interaction between both proteins occurs altering the conformation of the proteins. The E2b epitope also seems important for neutralization since the only serum not recognizing E2b (from animal 3.7) showed a significantly reduced neutralization titer against FeLV (fig. 4a, b).

Despite the similar localization and the limited sequence homology of these epitopes, neutralizing antisera against FeLV do not neutralize HIV (data not shown). In parallel, immunization with recombinant gp41 or epitopes recognized by broadly neutralizing antibodies such as 2F5 and 4E10 did not induce antibodies neutralizing HIV-1.

The results presented here show that immunizations with both the surface and the transmembrane envelope proteins (p15E and p45) induced higher titers of neutralizing antibodies suggesting that this combination may be the vaccination strategy of the future. This opinion is strengthened by the results of immunization studies using both envelope proteins of the murine leukemia virus [27, 28].

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