Induction of Neutralizing Antibodies Against the Transmembrane Envelope Protein gp41 of the Human Immunodeficiency Virus HIV-1

Stefan Langhammer et al., 2006, unpublished

Neutralizing antibodies such as 2F5 and 4E10 recognizing membrane proximal epitopes of gp41 have been isolated from HIV-infected patients. Since these epitope domains are highly conserved, the corresponding antibodies neutralize a broad range of HIV strains. Numerous attempts to generate 2F5-/4E10-like antibodies have failed, possibly because the conformation of the domain is difficult to reproduce. Recently we reported induction of neutralizing antibodies against the porcine endogenous retrovirus (PERV) and the feline leukaemia virus (FeLV) by immunization with their transmembrane envelope proteins p15E. Immunizing with a hybrid protein containing the 2F5-/4E10-epitopes of gp41 of HIV-1 and the N-terminal backbone of p15E of FeLV, we induced antibodies reacting with gp41 and neutralizing laboratory and primary strains of HIV-1. This strategy may be used to generate a vaccine inducing broadly neutralizing antibodies to prevent HIV infection.

Keywords: Vaccination, AIDS, Antigens/Peptides/Epitopes, Antibodies

40 million people are currently infected with HIV-1 worldwide. Immunization with an effective vaccine would be the best method to prevent further distribution of the virus. Although the correlate of protection is still unknown, the strategy to induce neutralizing antibodies has the great advantage of preventing infection and integration of the viral genome into the cellular genome, where the virus does persist. The induction of neutralizing antibodies against the transmembrane envelope protein gp41 of HIV-1 has the advantage that this protein contains highly conserved domains, which, due to their critical role during virus entry, cannot mutate (1). In contrast, the surface envelope protein gp120 is highly variable and first vaccine trials using gp120 antigens have failed (2). Indeed, antibodies recognizing conserved domains in gp41 (such as 2F5 and 4E10) and that neutralize a broad range of HIV strains have been isolated from HIV-infected individuals (3-5). However, most attempts to induce 2F5/4E10-like neutralizing antibodies by immunization with recombinant proteins or peptides containing the corresponding epitopes (ELDKWA in the case of 2F5 and NWFNIT in the case of 4E10) have failed (6-8). It remains unclear whether this is due to a complicated conformation that could not be reproduced in the antigens used for immunization, due to an extremely long CDR3 loop observed in both antibodies, 2F5 and 4E10 (9, 10) or whether both antibodies represent autoimmune antibodies specific for cardiolipin as proposed by Haynes et al (11) and are therefore difficult to induce.

Recently we induced antibodies neutralizing the porcine endogenous retrovirus (PERV) by immunization with its transmembrane envelope protein, p15E (12). Two epitopes were recognized by the p15E-specific goat antiserum, E1 and E2. E1 was located on the N-terminal helix, E2 in the same membrane proximal domain as the 2F5/4E10 epitope in gp41. Both epitopes should be located opposite, when the molecule was folded during the infection

process (12). We also induced antibodies neutralizing the feline leukaemia virus (FeLV) in goats and rats immunising them with p15E of FeLV (13). These sera also recognized two similarly located epitopes. Neutralizing antibodies recognizing the same epitopes were also induced by immunization with p15E of PERV in rats and p15E of FeLV in cats (13, 14). Despite their evolutionary distance, a sequence homology between PERV, FeLV and HIV exists in the E2 epitope (FEG**WF**N in the case of PERV, WFEG**WF**N in the case of FeLV and N**WF**NIT for the 4E10 epitope in HIV-1 gp41, identical amino acids in bold). In challenge experiments infecting cats with FeLV, which had been immunized with purified p15E of FeLV, we showed for the first time, that immunization with a transmembrane envelope protein protects from a retroviral infection *in vivo* (unpublished data).

In this report we used for immunization a hybrid protein based on the backbone of p15E of FeLV, in which the E2 domain was substituted by a corresponding E2 domain derived from gp41 of HIV-1 and containing the 2F5/4E10 epitope. Antibodies were induced neutralizing primary and laboratory strains of HIV-1. These data confirm similar findings, showing induction of neutralizing antibodies when immunizing with a hybrid protein composed of p15E of PERV, in which both E1 and E2 of gp41 (15, Fiebig et al., in preparation) or only E2 were introduced (16). We conclude that using such hybrid proteins broadly neutralizing antibodies may be generated and that these antigens should be an important component of an HIV vaccine.

Materials and Methods

Expression of the p15E/gp41 hybrid proteins

The p15E backbone was amplified from the pCaln-p15E vector (13) (aa476-512 and aa530-539), The DNA sequence of the immunosuppressive domain (17) and the peptides J and K (18) were deleted by two step PCR. The DNA sequence of the E2 region of HIV-1 IIIB gp41 was amplified from the pNL4-3 vector (p15E/gp41 I: aa645-aa680; p15E/gp41 II: aa654-aa680) (Fig. 1). Both amplified sequences were ligated using inserted restriction sites *Not*I, *Bam*HI and *Eco* RI and cloned into the pCal-n vector (Stratagene, Europe, Amsterdam, Netherlands). *E. coli* BL21DE3 cells were transformed and the hybrid proteins fused to the 4 kDa calmodulin binding protein (CBP) was produced.

Experimental animals and immunization

Wistar rats were obtained from a commercial supplier and housed in groups under barrier conditions. 10 rats were immunized intramuscularly (i.m.) twice (at 0 and 3 weeks unless stated otherwise) with 0.1 mg p15E/gp41 hybrid protein I in 0.5ml buffered saline emulsified in 1.2 ml Montanide® ISA 720 (Seppic, France, lot number 143521) (animals 1.1-1.7) or in 0.5ml Freund´s adjuvans incomplete (animals 1.8-1.10). 3 rats were immunized with the p15E/gp41 hybrid protein I and boostered once with 0.1mg gp41-derived peptide (aa656-680) emulsified in 0.25ml Montanide® ISA 720 (group 2) (Table 1). In addition 4 rats were immunized in the same way with 0.1 mg p15E/gp41 hybrid protein II and Montanide (group 3) ® ISA 720.

Virus neutralization assays

The virus stock for the neutralization assay was prepared as cell-free supernatant from C8166 cells infected with HIV-1IIIB and titrated on uninfected C8166 cells. 50µl cell-free virus-containing supernatant ($1x10^3$ $TCID_{50}$) were used to infect $5x10^4$ C8166 cells seeded into a well of a 96 U-well microtitre plate in a total volume of 100µl. Preimmune and immune sera were heat-inactivated at 56°C for 30 min. Serial dilutions of the sera were added to the virus and incubated for 45 min at 37°C before transfer to the cells. After 72 hours incubation

(37°C, 5% CO_2) cells were freeze-thawed three times and a lysis buffer containing 20 mg/ml of proteinase K in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 8.4) was added. The cells were incubated for 3h at 56°C followed by 10 min at 95°C to inhibit proteinase K activity. Provirus integration in infected cells was measured by quantitative real-time PCR as described below. Neutralization assays on macrophages with HIV-1 isolates Bal and SF162 were carried out on differentiated macrophages as described (19) and subsequently analyzed.

For infection of C8166 cells with PERV 50µl cell-free virus-containing supernatant of PERV/5°-infected 293 cells ($1 \times 10^{4.31}$ $TCID_{50}$/ml) (20) was added to the cells and identical procedures were carried out as described for HIV-1 neutralization assay, however using PERV-specific primers and probes.

Real time PCR

To analyze HIV-1 provirus integration 2µl of cell lysate from neutralization assays were used as template in a gp41-specific real-time PCR using primer SK68i (5′-GGARCAGCIGGAAGCACIATGG-3′), SK69i (5′-CCCCAGACIGTGAGITICAACA-3′) and the probe 6Fam-TGACGCTGACGGTACAGGCCAGAC-dabcyl (Taqman Universal Mastermix, Applied Biosystems). The assay was performed using a Stratagene MX4000 (55cycles, annealing at 55°C, no elongation phase) (21).

PERV provirus integration was analyzed using the forward primer 5′GTA CGT ACG TGG ATC CCT AAT CAC AGG ACC GCA ACA, the reverse primer 5′ACG TAC GTA CGA ATT CTC AGT TGA ACC ATC CTT AAA ACC and the probe 6-Fam-AGAAGGGACCTTGGCAGACTTTCT -dabcyl from the *pol* region of PERV. The settings were identical as described for the HIV-1-specifc real-time PCR. The neutralization efficacy was calculated as percentage of provirus integration comparing provirus integration in the presence of preimmune serum and immune serum. Therefore the Δct value of the control serum and the immune serum obtained from real time PCR was calculated and used in the

formula ($2^{-\Delta ct}$) x100%. The provirus integration in the presence of the preimmmune serum was set 100% by definition.

Electrophoresis and immunoblotting

SDS-PAGE and Western blotting were performed as described (22). 1 µg of the recombinant p15E/gp41 hybrid proteins I and II was used per lane. The monoclonal antibody 2F5 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and from Dr. Hermann Katinger.

Peptide ELISA

96 well plates were coated with 500ng/well of the gp41-derived peptide EKNEQELL**ELDKWA**SLW**NWFNIT**NWL (aa656-680, epitopes of 2F5 and 4E10 in bold) and preimmune and immune sera were applied in serial dilutions. ELISA was performed as described previously (13).

Purification of IgG from antisera by proteinG columns

Protein G columns (Montage®, Millipore) were used to purify IgG as described by the manufacturer.

Epitope mapping

Pepspot libraries of the entire gp41 of HIV-1IIIB and p15E of FeLV-A Glasgow strain were used (15-mer peptides overlapping by 13 amino acids, adsorbed on cellulose membranes, Jerini Biotools) and epitope mapping was performed as described previously (12, 13).

Results

Preparation of hybrid proteins for immunization

We have shown previously, that immunization with a hybrid protein containing p15E of PERV and two domains derived from gp41 of HIV, E1 and E2, induced HIV neutralizing antibodies (22). 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 also allows the induction of neutralizing antibodies specific for HIV-1. As additional modifications, 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 (Fig. 1A). The isu-domain is highly conserved amongst all retroviruses and was removed because synthetic peptides corresponding to this domain inhibit lymphocyte proliferation and modulate cytokine production (17, 23). In parallel, sequences corresponding to two other peptides (J and K), which were shown in previous immunization studies using different FeLV-derived peptides to induce enhancing antibodies (18), were removed (Fig. 1A). The proteins were purified by affinity chromatography (Fig. 1B) and used for immunization. Two hybrid proteins were generated, p15E/gp41 hybrid I with a size of 9.9kDa and p15E/gp41 hybrid II with a size of 8.9kDa both containing the 2F5/4E10 epitopes (Fig.1C) and the 4kDa calmodulin binding peptide (CBP) at their N-terminus. The p15E/gp41 hybrid proteins I and II were recognized by the mAb2F5 in Western blot analysis (Fig.1B) confirming the presence of the corresponding epitope in the proteins.

Hybrid proteins induced neutralizing antibodies

Rats were immunized twice with p15E/gp41 hybrid protein I (animals 1.1-1.7) and with using Montanide as adjuvans and with hybrid I using Freunds adjuvans (animals 1.8-1.10). Animals of group 2 were immunized with p15E/gp41 hybrid I and boostered with 0.1mg of a gp41-derived peptide containing the epitopes of 2F5 and 4E10. In addition, animals of group 3 were immunized twice with p15E/gp41 hybrid protein II and Montanide. The neutralizing efficacy of the sera was analyzed in neutralization assays using HIV-1IIIB and C8166 cells as well as the primary isolates HIV-1Bal and SF162 and freshly isolated human macrophages. Of 10 rats immunized and boostered with the hybrid protein I, 5 showed a neutralization of up to 99% of the HIV-1IIIB (Fig. 2A) with titers of neutralizing antibodies up to 1:16 (Fig. 2B). Using the primary isolates HIV-1Bal and HIV-1SF162 and freshly isolated human macrophages, two antisera (rats 1.2, 1.6) neutralised up to 81% at serum dilutions of 1:8 (Fig. 2C). In contrast to antisera from animals immunized with FeLV p15E/gp41 hybrid I, antisera from animals immunized and boostered with p15E/gp41 hybrid II or immunized with p15E/gp41 hybrid I and boostered with the gp41-derived peptide E2 did not significantly neutralised HIV-1IIIB (Fig. 2E).

Specificity of the HIV-1 neutralizing antibodies

To demonstrate that the neutralizing capacity was based on immunoglobulins, IgG were isolated from immune sera and tested. Neutralization up to 80% in comparison to purified IgG from preimmune serum was found (Fig. 3A), clearly indicating an immunoglobulin-mediated neutralization. To show specificity and to exclude a cytotoxic effect of the sera, in parallel the neutralizing effect on another retrovirus, PERV, infecting the same target cells,

C8166, was investigated. PERV infects human cells (24, 25), and it has been shown that PERV also infects C8166 cells (20). All sera did not inhibit PERV infection of C8166 cells (Fig. 3B), indicating specificity of the sera and absence of toxic effects.

Epitopes recognized by the neutralizing antisera

Analyzing the rat sera in an ELISA using a gp41-derived peptide containing the 2F5/4E10 epitope (EKNEQELL**ELDKWA**SLW**NWFNIT**NWL, 2F5 and 4E10 epitopes in bold), titers of binding antibodies ranging between 1:400 and 1:6400 were found (Table. 1), indicating the induction of gp41-specific antibodies. In order to identify the epitopes recognized by the neutralizing antisera, an epitope mapping was performed using pepspot membranes carrying overlapping peptides corresponding to the entire gp41 sequence of HIV-1 (Fig. 4A) and pepspot membranes carrying overlapping peptides corresponding to the ectodomain of p15E of FeLV-A (Fig. 4B). Using the gp41 peptides, epitopes such as QQEKNEQELL or EKNEQELLE were identified (Table 1) which were located C-terminal from the 2F5 epitope ELDKWA. In one case (rat 1.2) partially overlapping with the 2F5 epitope (QELL**ELDKW**) and in another case (rat 1.9) overlapping with both, 2F5 and 4E10 epitopes (**DKWA**SLW**NWFNI**) (Fig. 4C). In addition, one epitope was also detected on the p15E backbone (aa494-500: ALEESIS) (Fig. 4B). These data clearly demonstrate that immunization with a p15E/gp41 hybrid, containing the E2 domain of gp41 induced specific antibodies directed against the E2 domain of gp41 of HIV-1 able to neutralize HIV-1.

Discussion

The data obtained here indicates the possibility of induction of antibodies neutralizing HIV-1 of the type 2F5 and 4E10 using p15E of FeLV with the inserted E2 (2F5/4E10 epitope) domain of gp41 of HIV-1. This is the first report showing induction of 2F5/4E10-like neutralizing antibodies by a prime and a boost immunization with a recombinant protein based on a FeLV p15E backbone. However based on our results with immunization of p15E of PERV (12) Luo et al. (16) immunized with VSV recombinants expressing PERV p15E/gp41 hybrids and obtained neutralizing antibodies at a 1:20 serum dilution. However since they were able to show a 50% inhibition of HIV-1 HXB2 envelope virions by as low as 0.018µg/ml mAb2F5, it has to be assumed that their assay seems to be 100 times more sensitive than the HIV-1 neutralization assay used in the study presented here. We determined a 50% inhibition of HIV-1 IIIB by 2µg/ml mAb2F5.

In addition, a new epitope domain in gp41 recognized by the antisera induced by immunization with the p15E/gp41 hybrid protein was described (**QNQQEKNEQELL**ELDKW). The localization near the 2F5 epitope suggests that epitopes within this domain are the target of the neutralizing antibodies. However, either the recognition of a distinct epitope nor the data of the ELISA titres could be correlated with the neutralization efficacy of the sera obtained here. Thus it is likely that an antigen conformation has to be presented that might be formed in rats immunized with p15E/gp41 hybrid I and not in rats immunized with p15E/gp41 hybrid II. This indicates that the localization of the gp41 derived E2 domain is essential for the induction of neutralizing antibodies. It was also shown that a booster immunization with the hybrid protein I represents the better strategy than a peptide boost with the gp41 E2 peptide. Rats boostered with the E2 peptide did not show a significant neutralization of HIV-1 IIIB (Fig. 2, group 2).

In addition, one epitope was also detected on the p15E backbone (aa494-500: ALEESIS), corresponding to the epitope described for antisera induced by immunization of rats with p15E of FeLV and designated E1b (13). According to the protein folding model shown in Fig.1A which is based on previous publications demonstrating interactions between the N-terminal helix and the C-terminal helix of retroviral transmembrane envelope proteins (1, 26) the E1 epitope in the N-terminal helix is located opposite to the 2F5/4E10 epitope domain in the C-terminal helix. These findings correlate with our previously published data showing that antibodies neutralizing FeLV induced by immunization with its p15E recognized an N-terminal and a C-terminal epitope region in rats and in cats exposed in close proximity after interaction of both helices during intramolecular conformational changes (Fig. 5A).

Recently binding of 2F5 as well as 4E10 to cardiolipin was shown and a polyspecific autoreactivity was proposed for these antibodies (11). Although in preliminary experiments we were unable to confirm these data at least for 2F5, the newly induced neutralizing antibodies have to be investigated for their ability to bind cardiolipin. Most importantly, using a p15E/gp41 hybrid protein, antibodies of the 2F5/4E10-type neutralizing laboratory and primary strains of HIV-1 were easily induced, suggesting that they do not represent autoimmune antibodies and that the conformation of the antigen is of great importance. As gp41 derived peptides or gp41 proteins were not able to induce neutralizing antibodies against HIV-1 (6-8), it has to be assumed that the conformation of the hybrid antigen allows the induction of such antibodies. Our findings might offer a new way for proceeding towards an HIV-1 vaccine based on the induction of broadly neutralizing antibodies.

Acknowledgments

We thank Dr. S. Norley for critical reading of the manuscript and helpful advice and Andreas Müller and Martina Lau for technical assistance. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; the monoclonal antibody 2F5 from Dr. Hermann Katinger and C8166-45 cells from Dr. Robert Gallo.

References

1. Eckert, D.M., P.S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* 70, 777-810.

2. Francis, D.P., W.L. Heyward, V. Popovic, P. Orozco-Cronin, K. Orelind, Gee, C., A. Hirsch, T. Ippolito, A. Luck, M. Longhi, V. Gulati, N. Winslow, M. Gurwith, F. Sinangil, P.W. Berman. 2003. Candidate HIV/AIDS vaccines: lessons learned from the World's firstphase III efficacy trials. *AIDS* 17, 147-156.

3. Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, H. Katinger. 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J. Virol*. 67, 6642-6647.

4. Stiegler, G., R. Kunert, M. Purtscher, S. Wolbank, R.Voglauer, F. Steindl, H. A. Katinger.

2001. potent cross- clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* 17, 1757-1765.

5. Zwick, M., A. Labrijn, M. Wang, C. Spenlehauer, E. Saphire, J. Binley, J. Moore, G. Stiegler, H. Katinger, D. Burton and P. Parren. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1glycoprotein gp41. *J. Virol.*75, 10892-10905.

6. Tian, H., Y. Xiao, M. Zhu, Y.H. Chen. 2001. HIV epitope-peptides in aluminum adjuvant induced high levels of epitope-specific antibodies. *Int. Immunopharmacol.* 1, 763-768.

7. Lu, Y., Y. Xiao, J. Ding, M. P. Dierich, Y.H. Chen. 2000. Multiepitope vaccines intensively increased levels of antibodies recognizing three neutralizing epitopes on human immunodeficiency virus-1 envelope protein. *Scand. J. Immunol.* 51, 497- 501.

8. McGaughey, G.B., G. Barbato, E. Bianchi, R.M. Freidinger, V.M. Garsky, W.M. Hurni, J.G. Joyce, X. Liang, M.D., Miller, A. Pessi, J.W. Shiver, M.J. Bogusky. 2004. Progress towards the development of a HIV-1 gp41-directed vaccine. *Curr. HIV Res.* 2, 193-204.

9. Zwick, M.B., H.K. Komori, R.L. Stanfield, S. Church, M. Wang, P.W. Parren, R. Kunert, H. Katinger, I.A. Wilson, D.R. Burton. 2004. The long third complementarity-determining region of the heavy chain is important in the activity of the broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2F5. *J. Virol.* 78, 3155-

3161.

10. Cardoso, R.M., M.B. Zwick, R.L. Stanfield, R. Kunert, J.M. Binley, H. Katinger, D.R. Burton, I.A. Wilson. 2005. Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity*. 22, 163-173.

11. Haynes,B.F., J. Fleming, E.W. St Clair, H. Katinger, G. Stiegler, R. Kunert, J. Robinson, R.M. Scearce, K. Plonk, H.F. Staats, T.L. Ortel, H.X. Liao, S.M. Alam. 2005. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* , 308,1906-1908.

12. Fiebig, U., O. Stephan, R. Kurth, J. Denner. 2003. Neutralizing antibodies against conserved domains of p15E of porcine endogenous retroviruses: basis for a vaccine for xenotransplantation? *Virology* 307, 406-413.

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

14. Langhammer S, Hübner J, Kurth R, Denner J. 2005. Antibodies neutralising feline leukemia virus (FeLV) in cats immunised with the transmembrane envelope protein p15E. *Immunology* Epub ahead doi: 10.1111/j.1365-2567.2005.02291.x

15. Fiebig U, Schmolke M, Karlas A, Kurth R, Denner J. 2005. Induction of anti-viral neutralising antibodies, World Intellectual Property Organisation patent WO 2005/021574.

16. Luo, M., F. Yuan, Y. Liu, S. Jiang, X. Song, P. Jiang, X. Yin, M. Ding, H. Deng. 2005. Induction of neutralizing antibody against human immunodeficiency virus type 1 (HIV-1) by immunization with gp41 membrane-proximal external region (MPER) fused with porcine endogenous retrovirus (PERV) p15E fragment. *Vaccine*. [Epub ahead of print]

17. Denner, J. 1998. Immunosuppression by retroviruses: implications for xenotransplantation. *Ann. N. Y. Acad. Sci.* 862, 75-86.

18. Nick, S., J. Klaws, K. Friebel, C. Birr, G. Hunsmann, H. Bayer. 1990. Virus neutralizing and enhancing epitopes characterized by synthetic oligopeptides derived from the feline leukaemia virus glycoprotein sequence. *J. Gen. Virol.* 71, 77-83.

19. Ruppach, H., P. Nara, I. Raudonat, Z. Elanjikal, H. Rubsamen-Waigmann, U. Dietrich. 2000. Human immunodeficiency virus (HIV)-positive sera obtained shortly after seroconversion neutralize autologous HIV type 1 isolates on primary macrophages but not on lymphocytes. *J. Virol.* 74, 5403-5411.

20. Denner J, Specke V, Thiesen U, Karlas A, Kurth R. 2003. Genetic alterations of the long terminal repeat of an ecotropic porcine endogenous retrovirus (PERV) during passage in human cells *Virology*;314:125-33.

21. Schweiger, B., G. Pauli, H. Zeichhardt, C. Kücherer. 1997. A multicentre quality assessment study to monitor the performance of HIV-1 PCR. *J. Virol. Methods* 67, 45-55.

22. Tacke, S.J., K. Bodusch, A. Berg, J. Denner. 2001. Sensitive and specific immunological detection methods for porcine endogenous retroviruses applicable to experimental and clinical xenotransplantation. *Xenotransplantation* 8, 125-135.

23. Cianciolo, G.J., T.D. Copeland, S. Oroszlan, R. Snyderman. 1985. Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. *Science* 230, 453-455.

24. Patience C Takeuchi Y, Weiss RA. 1997. Infection of human cells by an endogenous retrovirus of pigs. *Nature Medicine* 3, 282

25. Specke V, Rubant S, Denner J.2001. Productive infection of human primary cells and cell lines with porcine endogenous retroviruses. *Virology* 285, 177-180.

26. Gallo, S.A., C.M. Finnegan, M. Viard, Y. Raviv, A. Dimitrov, S.S. Rawat, A. Puri, S. Durell, R. Blumenthal. 2003. The HIV Env-mediated fusion reaction. *Biochem. Biophys.Acta* 1614, 36-50.

TABLE I. Characterization of sera induced with p15E/gp41 hybrid proteins I and II

Rat	Prime, Boost[a]	Elisa Titre[b]	Neutralization titre in RTQ	Neutralization in RTQ[c]	Epitope on C-terminal gp41
1.1		$4x10^2$	0	0%	QQEKNEQE
1.2		$6.4x10^3$	**1:16**	**95%**	QELL**ELDKW**
1.3		$1.6x10^3$	**1:8**	**70%**	QQEKNEQE
1.4		$6.4x10^3$	0	0%	EKNEQELL**E**
1.5	0.1mg	$6.4x10^3$	0	0%	QNQQEKNE
1.6	p15E/gp41	$6.4x10^3$	**1:16**	**99%**	QNQQEKNE
1.7	hybrid I	$1.6x10^3$	**1:16**	**85%**	EKNEQELL**E**
1.8		$1.6x10^3$	0	0%	**ELDKWA**SLW
1.9		$1.6x10^3$	**≥ 1:4**	**90%**	**DKWA**SL**WNFN**
1.10		$1.6x10^3$	0	0%	NEQELL**EL**
2.1	0.1mg	$1.6x10^3$	0	0%	QNQQEKNE
2.2	p15E/gp41	$1.6x10^3$	0	0%	EKNEQELL**E**
2.3	hybrid I,	$1.6x10^3$	0	0%	EKNEQELL**E**
2.4	E2-peptide	$1.6x10^3$	0	0%	n.d
3.1	0.1mg	n.d	0	0%	n.d
3.2	p15E/gp41	n.d	0	0%	n.d
3.3	hybrid II	n.d	0	0%	n.d
3.4		n.d	0	0%	n.d

[a] Animals 1.1-1.7, and animals from groups 2 and 3 were immunized with Montanide ISA 720, animals 1.8-1.10 were immunized with Freund´s adjuvant

[b] Peptide-ELISA using peptide HIV-1 E2 (EKNEQELL**ELDKWA**SLW**NWFNIT**NWL)

[c] Neutralization in percentage obtained at a serum dilution of 1:4

Figures

FIGURE 1. Characterization of the p15E/gp41 hybrid proteins. A, Schematic presentation of the p15E ectodomain of FeLV and the resulting modified p15E/gp41 hybrid protein. The localization of the immunosuppressive (isu) domain, the peptides J and K, the cystein-cystein loop (Cys-loop) as well the N- and C-helix regions (NHR, CHR) and the epitope domains (E1, E2) are indicated. The white epitope domain E2 indicates the C-terminal membrane proximal gp41 sequence. B, Evidence by a Western blot analysis using the mAb 2F5 (0.5mg/ml), that the 2F5 epitope is introduced into the p15E/gp41 hybrid protein. 1 - hybrid protein I, 2 - hybrid protein II, 1µg of protein was loaded. C, Amino acid sequence of the p15E/gp41 hybrid I and the p15E/gp41 hybrid II proteins. The C-terminal gp41 E2 sequence is printed in bold, the 2F5 and 4E10 epitopes are boxed.

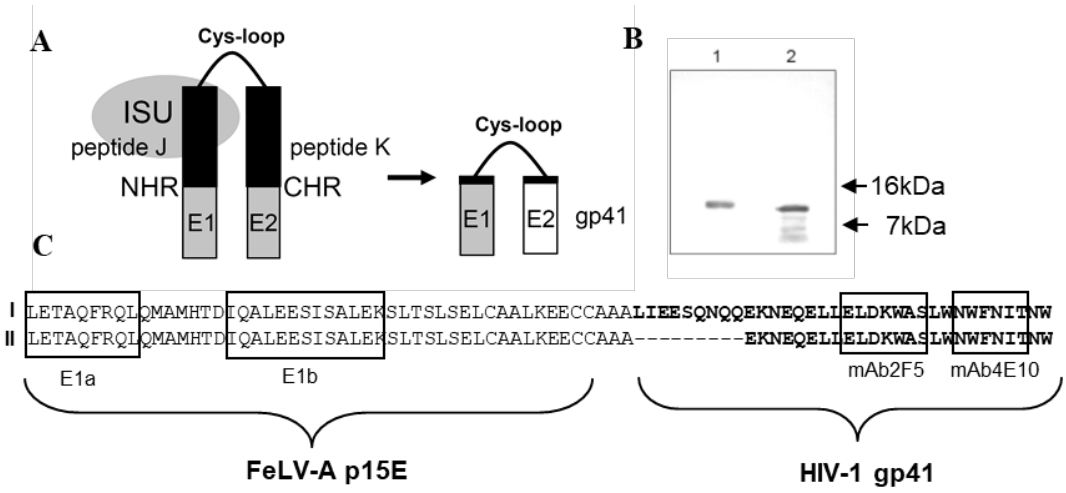

FIGURE 2. Neutralization capacity of rat antisera induced by immunization with the p15E/gp41 hybrid proteins I and II. HIV-1 infection was measured as provirus integration by quantitative real time PCR. The mAb 2F5 was used as positive control at a concentration of 100µg/ml. A pool of preimmune sera was used as negative control. Provirus integration in the presence of the preimmune serum was taken as 100%. A, Neutralization assay using HIV-1IIIB and C8166 cells, all sera were added at concentration 1:4. B, Neutralization assay using HIV-1IIIB and C8166 cells, serum 1.2 was added at different dilutions. C, D, Neutralization assay using the primary HIV-1 isolates SF162 (C) and Bal (D) and freshly isolated macrophages. Rat immune sera 1.2 and 1.6 were used at a dilution 1:8. E, Neutralization assay using HIV-1IIIB and C8166 cells, rat sera of animal groups 2 and 3 which differed in the boost immunization (boost with peptide E2 versus p15E/gp41 hybrid II) were added at a concentration 1:4.

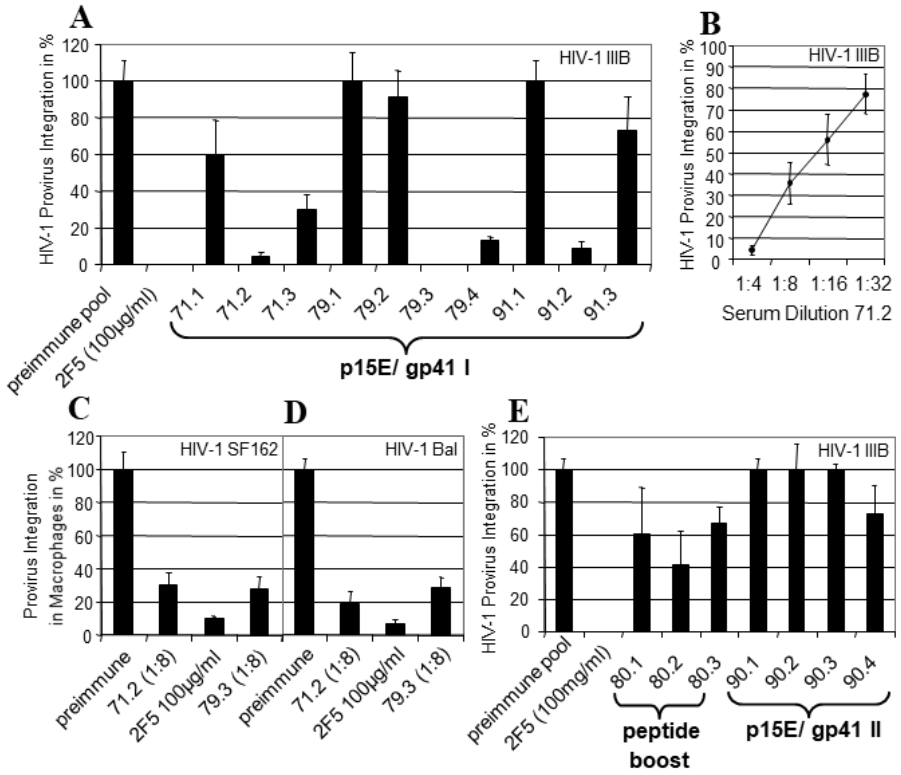

FIGURE 3. Neutralization of HIV-1 is mediated by immunoglobulins and is virus specific. A, Neutralisation assay using HIV-1 IIIB and C8166 cells, immune and preimmune sera of rat 1.9 were used at a dilution 1:4. Immunoglobulins purified by affinity chromatography were used at the same dilution as the serum. B, Neutralization assay using HIV-1 IIIB and C8166 cells as well as PERV and C8166 cells, serum from rat 1.7 was dialyzed, concentrated twofold and added at different concentrations.

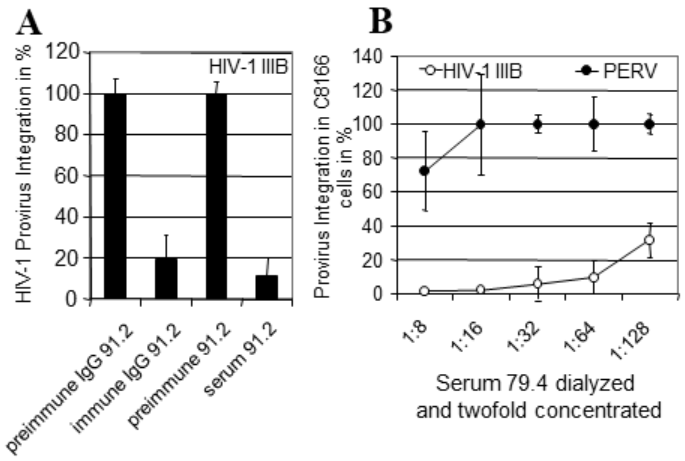

FIGURE 4. Epitope mapping using pepspot membranes with overlapping peptides and rat sera after immunization with the p15E/gp41 hybrid protein. A, Results obtained for the neutralizing rat serum 1.2 using overlapping peptides corresponding to the entire gp41 of HIV-1. The serum was used at 1:500 and an ECL method was used for detection. The sequences of the reacting peptides were shown and the identified epitopes were framed. B, Results obtained for the same serum and overlapping peptides corresponding to the entire p15E of FeLV, the serum was used at 1:500. C, Summary of the epitope mapping of all rat antisera using gp41-derived peptides. The sequence of the E2 domain used for immunization as part of the p15E/gp41 hybrid I is shown, strong epitopes are drawn in black, weak in grey, the epitopes of 2F5 and 4E10 are shown in light grey.

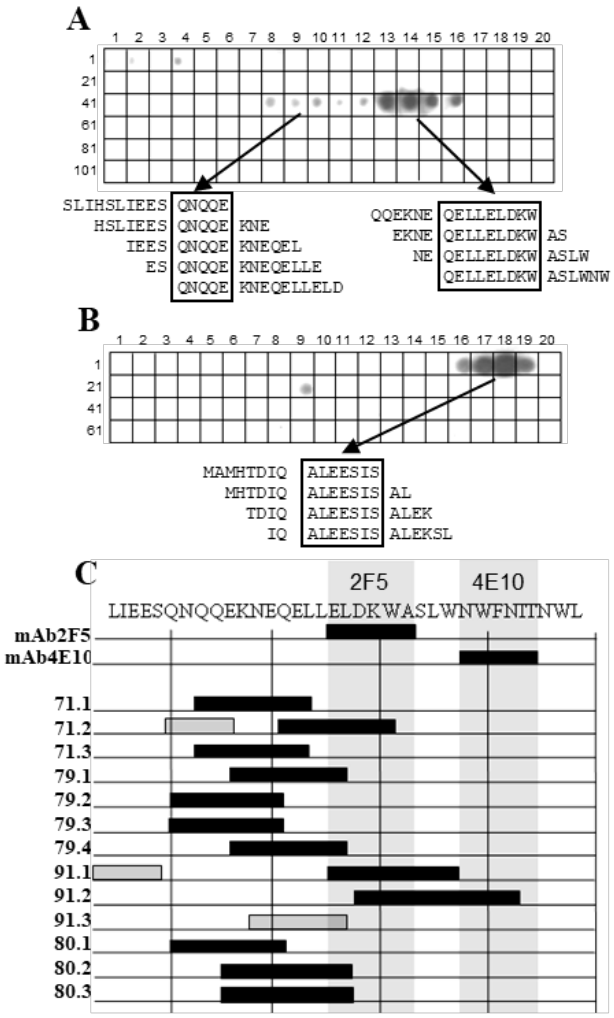

FIGURE 5. Comparison of the localization of epitopes recognized by sera after immunization of rats and cats with p15E of FeLV-A with the localization of epitopes recognized by the broadly HIV neutralizing antibodies 2F5 and 4E10 isolated from HIV-1 infected individuals. A, Epitopes on the transmembrane envelope protein p15E of FeLV recognized by neutralizing antibodies induced in rats (13) and cats (14), B, 2F5 and 4E10 epitopes on gp41 of HIV-1 (3, 5). The transmembrane envelope proteins were drawn in a folded form after interaction between the N-terminal helix region (CHR) and the (NHR) (framed). The E1 domain in gp41 of HIV-1 corresponds to a peptide enhancing the binding of 2F5 and 4E10 to their epitopes (15, see discussion).