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VIROLOGY

Virology 307 (2003) 406–413

www.elsevier.com/locate/yviro

Neutralizing antibodies against conserved domains of p15E of porcine endogenous retroviruses: basis for a vaccine for xenotransplantation?

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Received 16 August 2002; returned to author for revision 3 October 2002; accepted 8 November 2002

Abstract

Porcine xenotransplants may offer a potential solution to the problem posed by the limited supply of allotransplants. However, xenotransplantation may be associated with the risk of transmission of microorganisms, in particular of porcine endogenous retroviruses (PERVs) that are an integral part of the porcine genome and able to infect human cells in vitro. Possible strategies to prevent virus transmission include the development of PERV knockout animals or of effective vaccines. When antisera prepared against the main structural proteins of PERV were screened, a goat antiserum against the recombinant ectodomain of the transmembrane envelope protein p15E was found to neutralize PERV infectivity. Epitope mapping using overlapping peptides revealed two epitopes, E1 (GPQQLEK) and E2 (FEGWFN). These sequences are identical for all PERVs and are highly conserved among all gammaretroviruses. Interestingly, antibodies isolated from AIDS patients and specific for sequences of HIV-1 partially homologous with E2 (Mab4E10, LWNWFN) or located in close proximity to E2 (Mab2F5, ELDKWA) are known to neutralize several strains of HIV-1. It is the first report showing epitope mapping of gammaretrovirus-specific neutralizing antibodies and demonstrating similarity to corresponding epitopes in HIV. These domains of the transmembrane proteins of different retroviruses are an effective target for neutralizing antibodies and may be a useful antigen to create an antiretroviral vaccine.

Keywords: Retroviruses; Xenotransplantation; Vaccine; Neutralizing antibodies; Epitope mapping

Introduction

Xenotransplantation is a possible solution to overcoming the shortage of allografts for transplantation. Pigs are currently the most favored donors of organs and tissues for xenotransplantation. However, the use of organs across the species barrier may be associated with the risk of transmission of microorganisms, especially porcine endogenous retroviruses (PERVs), which are an integral part of the porcine genome (Patience et al., 2001) and infect human cells in vitro (Patience et al., 1997; Specke et al., 2001a). It is still unknown whether PERVs can infect human transplant recipients in vivo and, if so, whether they are pathogenic. PERVs are closely related to retroviruses such as the feline and murine leukemia viruses, which induce in the infected host leukemias and immune deficiencies (Denner, 1987).

There are several possible approaches to preventing PERV transmission to the transplant recipient. First, animals may be produced which do not release human-tropic infectious virus, either by conventional breeding (Oldmixon et al., 2002) or by using genetic knockout technologies already developed in pigs to reduce the hyperacute rejection against galactose-α1,3-galactose epitopes (Dai et al., 2002; Lai et al., 2002).

Many of the more than 50 proviral copies located in the pig genome are unable to produce infectious virus due to deletions and mutations. Such defective viruses have been found in many other species, including man (Löwer et al., 1996). However, infectious particles may be produced not only by replication-competent viruses, but also as the result of recombination and/or complementation of intact genes from defective proviruses.

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doi:10.1016/S0042-6822(02)00140-X

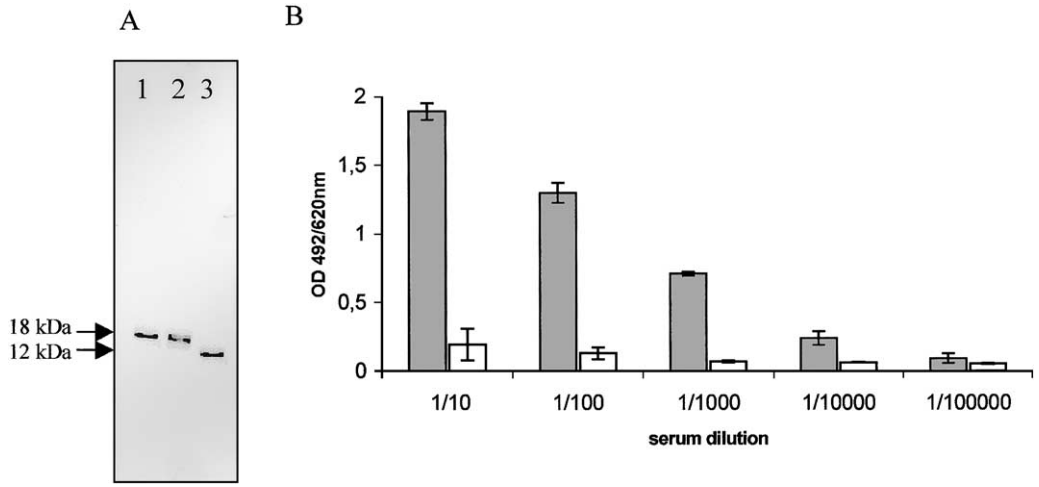

Fig. 1. Reactivity of a goat antiserum specific for PERV p15E. (A) Western blot analysis using highly purified PERV (1), highly purified viral p15E (2), or the recombinant ectodomain of p15E as used for immunization (3). The molecular mass of the proteins is indicated on the left. (B) Determination of ELISA titers using the recombinant ectodomain of p15E: gray columns, immune serum; white columns, preimmune serum.

Second, as transplantation is usually a medical intervention planned a long time in advance, it would be feasible to vaccinate transplant recipients against PERV. While the development of a vaccine against lentiviruses such as HIV has not been very successful to date (Liu, 1998; Schultz and Stott, 1994), effective vaccines against type C retroviruses (now termed gammaretroviruses), such as MuLV and FeLV, exist (Lee et al., 1977; Montelaro and Bolognesi, 1995; Peters et al., 1975). Leukemia-preventing antibodies were predominantly induced by immunization with recombinant envelope proteins gp 70 and p15E (Marciani et al., 1991). However, none of the five commercial FeLV vaccines currently available in the USA and Europe provide 100% protection against infection. Three are composed of inactivated whole virus and two are gp70 subunit vaccines (Marciani et al., 1991; Sparkes, 1997). Since PERVs are gammaretroviruses closely related to MuLV and FeLV, an effective vaccine against PERV may be a real possibility.

Here we describe an antiserum specific for the transmembrane envelope protein p15E of PERV with strong neutralizing activity. This antiserum was developed for several reasons: (i) To develop an approach to immunization against and prevention of PERV transmission in xenotransplantation. The transmembrane envelope (TM) protein was used because we intended to create a vaccine neutralizing the virus but not being harmful for the transplant and only p15E has conformational intermediates not present on the budding or free virus but generated during infection (fusion-competent conformations). (ii) To evaluate our immunological detection methods, such as Western blot assays and ELISAs (Tacke et al., 2001), and as additional tool to study the immunosuppressive properties of p15E (Denner, 1997, 1998; Tacke et al., 2000). (iii) To study this immunization approach and to apply it—if possible—to other retroviruses, including HIV, since broadly neutralizing antibodies directed against the TM protein of HIV-1 were described (Muster et al., 1993). Interestingly, the epitopes recognized by the PERV-specific serum are located in a domain of p15E that is highly conserved among gammaretroviruses and which shares homology with regions of the HIV-1 gp41 known to induce neutralizing activity against different clades of HIV-1 (Zwick et al., 2001). This domain may therefore serve as a basis for the development of preventive vaccines to block PERV infection in xenotransplant recipients. The data presented here may have implications for antiretroviral vaccine development in general.

Results

Reactivity of the p15E-specific antiserum

In order to generate a p15E-specific antiserum, the recombinant ectodomain of p15E (amino acids 488–597) was produced using a cloned PERV-A sequence derived from PERV-producing PK-15 pig kidney cells by PCR amplification. The protein was successfully expressed as a calmodulin binding peptide(CBP) fusion protein in *Escherichia coli* and purified by CBP affinity chromatography. The protein had a molecular mass of 12 kDa compared to the 18 kDa of viral p15E (Fig. 1A). Immunization of a goat yielded antiserum recognizing p15E in purified virus preparations, purified viral p15E, and the recombinant protein used for immunization as shown by Western blot analysis (Fig. 1A). It also reacted with the recombinant p15E in an ELISA, but it did not contain antibodies directed against the immunosuppressive domain of p15E thought to be involved in retrovirus-induced immunosuppression (Denner, 1998). The preimmune serum did not react in any assay (Fig. 1B).

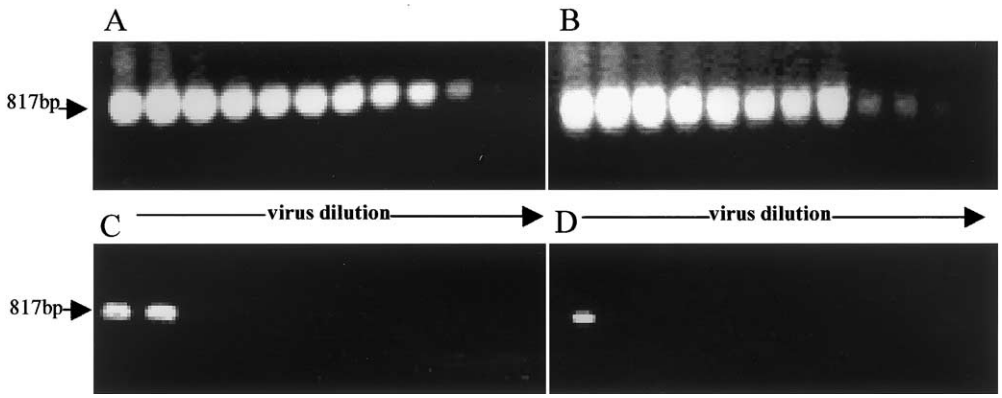

Fig. 2. Neutralization of PERV/5° by the goat antiserum specific for p15E. Infection was measured by PCR detecting provirus integration. Virus-containing supernatant was diluted 1:2 in the absence of serum (A), in the presence of preimmune serum diluted 1:5 (B), in presence of the immune serum diluted 1:5 (C), and in the presence of affinity-purified antibodies (100 μg/ml) (D). The size of the amplicon (817 bp) is indicated on the left.

Neutralizing activity of the p15E-specific antiserum

Heat-inactivated (56°C, 30 min) serum was shown to inhibit infection of human 293 kidney cells by PERV/5°, a recombinant virus with the PERV-A receptor binding site (Fig. 2). This virus, generated by serial passage of PERV-NIH/3° (Wilson et al., 1998, 2000) on human 293 kidney cells, is characterized by high infectivity and genetic alterations in the LTR (Denner et al., 2001, 2002). The preimmune serum had no neutralizing activity. When antibodies purified by affinity chromatography using immobilized p15E were used in the neutralization assay, a clear inhibition was seen (Fig. 2). This indicates that the neutralizing activity is based on antibodies, not on complement or other soluble antiviral factors.

Epitope mapping

To analyze the epitopes against which the neutralizing antibodies were directed, linear 13-mer peptides overlapping by 11 amino acids and corresponding to the entire sequence of p15E of PERV-A were used. These peptides were bound covalently by the C-terminus to a cellulose membrane. Two main epitopes were found (Fig. 3A), one located at the N terminus (E1, GPQQLEK) and the other at the C terminus (E2, FEGWFN). As expected, these epitopes were part of the sequence used for immunization (Fig. 3B). The sequences corresponding to the epitopes are conserved among all PERVs (PERV-A, PERV-B, PERV-C) and are homologous to sequences in the transmembrane envelope protein of other gammaretroviruses and partially related to corresponding sequences in the transmembrane envelope protein of lentiviruses such as HIV-1 (Fig. 3C).

Antibodies purified by affinity chromatography using immobilized p15E reacted in an ELISA with the peptides E1, E2, E1 and E2, as well as with purified recombinant p15E (Fig. 4). They did not react with the immunosuppressive (isu) peptide, indicating the specificity of the reaction. It remains unclear whether the additive effect when both peptides were added simultaneously is due to a reaction with a discontinuous epitope.

Inhibition of the neutralizing activity with peptides corresponding to the epitopes E1 and E2

To determine whether antibodies binding to the epitopes mapped were indeed responsible for the neutralizing activity, inhibition studies were performed. Affinity-purified neutralizing antibodies were preincubated with recombinant p15E and with both synthetic peptides. An inhibition of neutralization was observed when p15E, E1, and E2 were added (Fig. 5). No inhibition was seen when the control isu peptide was added. These data indicate that antibodies specific for both epitopes are responsible for the neutralizing activity.

Discussion

Xenotransplantation represents one approach to overcoming the shortage of organs required to treat patients with severe organ failure. In Germany, for example, 550 heart and 2300 kidney transplantations are performed annually. However, over 2000 heart and 11,000 kidney patients remain on the waiting list, of whom 25% die before receiving the lifesaving organ. Alternative approaches include the prevention of those diseases leading to the loss of organ function or the generation of new organs from stem cells. Since stem cell research is in its infancy, xenotransplantation seems at present to be the only reasonable approach. However, before xenotransplantation becomes a clinical reality, three main criteria have to be met: prevention of immunological rejection, physiological compatibility, and microbiological safety. Whereas most microorganisms of the pig that may infect humans and lead to xenozoonoses may be eliminated by breeding of specified pathogen-free (SPF) animals, PERVs cannot be eliminated so easily.

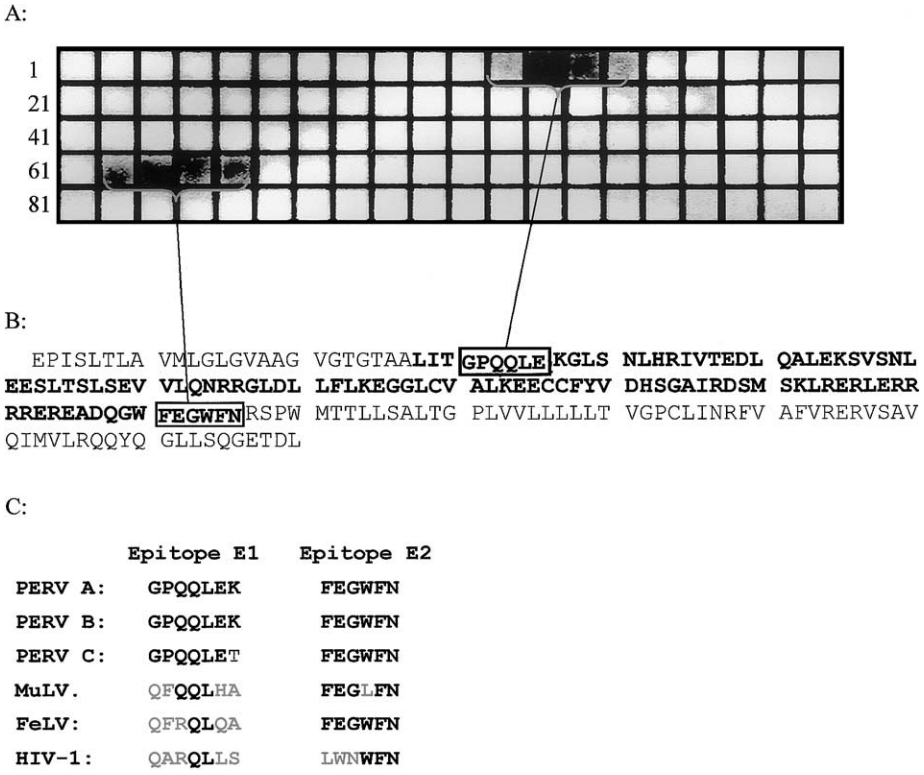

Fig. 3. Epitope mapping of neutralizing antibodies specific for PERV p15E. The result of the dot blot ECL method using overlapping peptides is given in A. In B the sequence of p15E of PERV-A is given, the sequence of the recombinant protein used for immunization is printed in bold, and the epitopes are framed. In addition, a comparison of the sequence of the epitopes with corresponding sequences of other retroviruses is given in C.

Although the knockout technology is established for pigs (Lai et al., 2002; Dai et al., 2002), it remains unclear whether the elimination of replication-competent human-tropic viruses would be sufficient or whether the remaining non-replication-competent viruses may complement each other. In addition, recombinations between human-tropic and ecotropic PERVs have often been observed when pig lymphocyte-derived PERVs infect human cells (Wilson et al., 1998, 2000). Therefore the generation of a vaccine may be an appropriate solution to preventing PERV transmission to human recipients. Such a vaccine should be directed against all PERVs produced by pig cells and should not harm the transplant if the virus is expressed or budding on the cell surface.

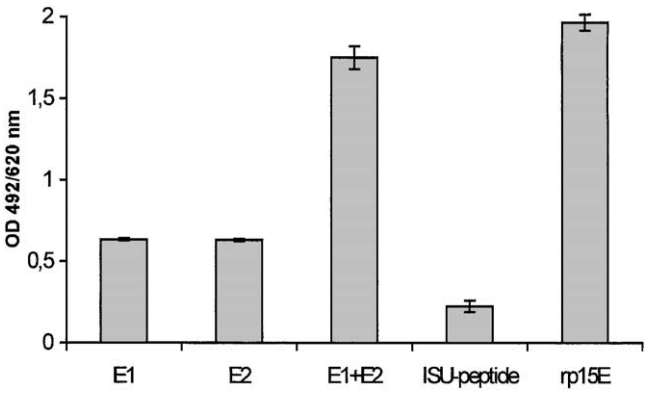

Fig. 4. ELISA reactivity of the PERV p15E-specific antiserum specific using recombinant p15E and synthetic peptides corresponding to the mapped E1 and E2 epitopes. As a control a peptide corresponding to the immunosuppressive (isu) domain was used.

The generation of an antiretroviral vaccine is also of broad academic and public interest. The urgent need for the production of vaccines against retroviruses was initiated by the discovery that members of this family cause leukemia and acquired immune deficiency syndrome (AIDS) in humans. It is well known that the HIV epidemic represents a transpecies transmission of SIV in vivo (Gao et al., 1999), and PERVs may also therefore be a risk for patients and contact persons during xenotransplantation. The potential for transspecies transmission may be enhanced by the absence of normal antiviral immune responses resulting from the pharmacological immunosuppression needed to prevent transplant rejection. Although in vivo transspecies transmission of PERV was recently claimed in SCID mice (Deng et al., 2000; van der Laan et al., 2000) other attempts to infect immunosuppressed small animals and nonhuman primates failed (Denner et al., 2001; Specke et al., 2002, 2001b). In addition, no evidence for PERV infection was found in experimental pig-to-human xenotransplantations, including ex vivo perfusion (Paradis et al., 1999; Tacke et al., 2001). The limited contact with porcine tissues, the small number

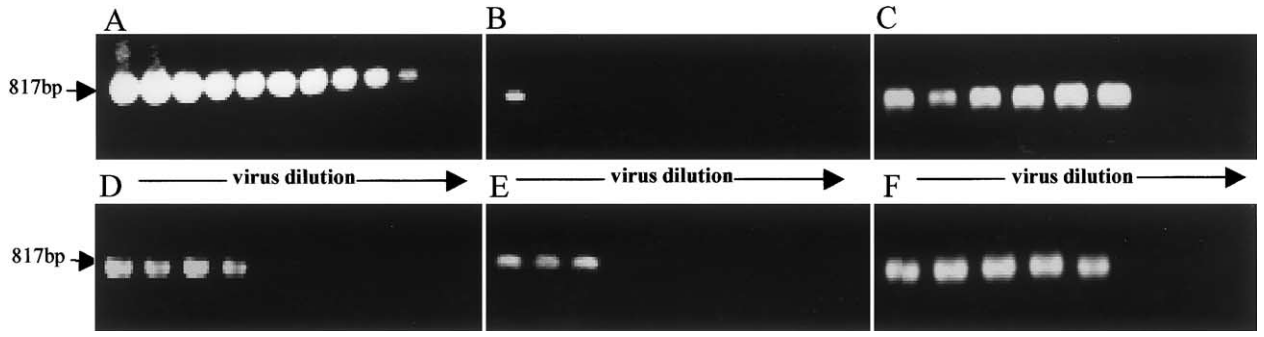

Fig. 5. Inhibition of the neutralizing activity of the antiserum specific for PERV p15E. Virus was diluted 1:2 in the absence of serum (A) or in the presence of 100 μg/ml of affinity chromatography-purified neutralizing antibodies (B–F). (B) Shown are dilution of the virus without inhibitor (with medium alone), (C) the inhibition of the neutralizing activity by 25 μg/well of recombinant p15E, (D) by the synthetic peptide corresponding to the epitopes E1 (25 μg/well), (E) to the epitope E2 (25 μg/well), and (F) by a mixture of E1 and E2 (12.5 μg/well + 12.5 μg/well).

of pig cells, and the absence of strong pharmacological immunosuppression might have contributed to the lack of PERV transmission. Therefore the possibility that extended contact with the implanted porcine cells or inoculation of larger amounts of PERV could result in an infection cannot be excluded.

Vaccines against gammaretroviruses related to PERV, such as FeLV and MuLV, exist, but their efficacy should be improved (Sparkes, 1997). Several regions that elicit FeLV-neutralizing antibodies were defined on the outer membrane protein gp70 and on the transmembrane protein p15E (Elder et al., 1987). It was shown that immunization with gp70 and p15E is more effective than immunization with gp70 alone (Schwarz et al., 1984). Interestingly, one of the peptides inducing neutralizing antibodies, 17B (Elder et al., 1987), derived from the C-terminal transmembrane region of p15E of FeLV, contains the epitope E2 (FEGWFN) described here. This epitope is highly conserved among gammaretroviruses. Peptid C18B, derived from the N-terminal region near the fusion peptide, also induces neutralizing antibodies and contains a sequence corresponding to epitope E1. Both peptides had been conjugated to keyhole limpet hemocyanin for immunization of rabbits; however, no epitope mapping of the neutralizing sera was performed.

At present, the main goal of many researchers is the generation of an AIDS vaccine. Numerous attempts to generate such a vaccine have failed (Montelaro and Bolognesi, 1995). However, broadly neutralizing monoclonal antibodies against the TM protein gp41 of HIV-1 have been prepared from HIV-1-seropositive individuals (Muster et al., 1993; Zwick et al., 2001). Both monoclonal antibodies (Mab 2F5/Mab 4E10) recognize a relatively conserved region in gp41 close to the viral membrane (Fig. 6). However, immunization with synthetic peptides corresponding to the sequence of 2F5 or 4E10 did not result in neutralizing antibodies (Liao et al., 2000; Lu et al., 2000; Tian et al., 2001; Xiao et al., 2000), suggesting some conformational requirements of the vaccine (Zwick et al., 2001).

The conformation of epitopes in the transmembrane envelope protein of different retroviruses recognized by neutralizing antibodies is unclear, although results suggest that possibly the neutralizing antibodies bind to a discontinuous epitope. Being conserved, the C-terminal extracellular region is an obvious candidate for inducing broadly reactive neutralizing antibodies. There are two mechanisms by which the neutralizing activity may be explained. First, after binding of the viral surface envelope protein gp70 to its corresponding (yet unknown) receptor and conformational changes in the transmembrane envelope protein p15E the interaction of the C-terminal helix with the N-terminal helix of p15E brings the epitopes E1 and E2 in close proximity and the neutralizing antibodies may bind to the epitopes E1 and E2 and prevent fusion of the viral membrane with the cellular membrane. Second, despite the fact that most of the transmembrane envelope protein is occluded by the surface envelope protein gp70, the domains corresponding to E1 and E2 may be free, allowing binding of the neutralizing antibodies that then prevent, due to their size, the binding of the surface envelope protein to the receptor. The latter

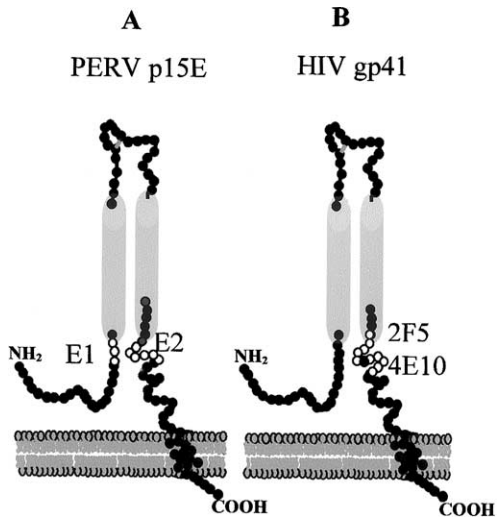

Fig. 6. Localization of the epitopes E1 and E2 in p15E of PERV (A) and localization of epitopes in gp41 of HIV-1 recognized by neutralizing antibodies in HIV-infected individuals (B).

mechanism was also proposed by Zwick et al. (2001) for neutralization of HIV by Mab 2F5 and Mab 4E10.

Experiments are under way to reproduce the induction of such neutralizing antibodies against PERV as a first step toward the generation of a vaccine that may prevent virus infection but does not harm the xenotransplant as well as to apply the same immunization protocol to obtain neutralizing antibodies against HIV-1.

Materials and methods

Cells and viruses

PERV/5° and human kidney 293 cells were used for titration studies. PERV/5° was derived from PERV-NIH/3° (kindly provided by C. Wilson, FDA, Bethesda, MD) (Wilson et al., 1998, 2000) by serial passages on human 293 cells. PERV/5° is characterized by genetic changes in the LTR and a higher infectious yield in comparison to PERV-NIH/3° (Denner et al., 2001, 2002).

Generation of recombinant p15E

DNA from PERV-producing PK-15 pig kidney cells was isolated using a Qiagen DNA isolation kit. Using the forward primer 5′GTA CGT ACG TGG ATC CCT AAT CAC AGG ACC GCA ACA and the reverse primer 5′ACG TAC GTA CGA ATT CTC AGT TGA ACC ATC CTT AAA ACC A a sequence corresponding to the ectodomain of the transmembrane envelope protein p15E (amino acids 488–597) was amplified by polymerase chain reaction and cloned into the pCal-n vector (Stratagene, Europe, Amsterdam, Netherlands). *E. coli* BL21 DE3 cells were transformed and rp15E N-terminally fused to a 4 kDa CBP was produced. The fusion protein was purified by calmodulin resin affinity chromatography (Stratagene). For immunization and for inhibition experiments the protein was extensively dialyzed against phosphate-buffered saline (PBS).

Immunization

To generate rp15E-specific antibodies, a goat was inoculated im twice (0 and 4 weeks) with 0.5 mg of the affinity-purified recombinant fusion protein emulsified in Freund's adjuvant.

Affinity chromatography of neutralizing antibodies

Purified rp15E was coupled to CNBr-activated Sepharose (Pharmacia) and specific antibodies were eluted using Tris–glycine buffer, pH 2.3. The antibodies were dialyzed against PBS.

Purification of viral p15E

To purify viral p15E, PERV/5° particles were pelleted and purified by sucrose centrifugation. The particles were freeze-thawed twice and than dissolved in TEN buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.4) with 0.1% Triton X-100. The lysate was centrifuged at 100,000 *g* for 2 h at 4°C to remove insoluble material. The second step of the purification procedure consisted of immunoaffinity chromatography. Purified antibodies from the goat anti-p15E serum were coupled to NHS-activated Sepharose 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden). The viral lysate was applied to the immunoaffinity column preequilibrated in TEN with 0.1% Triton X-100. After washing with the same buffer, p15E was eluted with 0.5 M propionic acid/0.1% Triton X-100 (pH 2.5) and the eluant was collected into tubes containing 2 M Tris–0.1% Triton X-100 (pH 7.6). The purity was verified by SDS–PAGE and silver staining.

Peptides and ELISA

The following peptides were used: the immunosuppressive peptide of PERV-A (isu-peptide), synthesized as described (Denner et al., 1994; Tacke et al., 2000) and E1 and E2 (both synthesized by Jerini Biotools, Berlin, Germany). Peptide ELISA was performed as described (Denner et al., 1994); 1 μg per well of peptide or recombinant p15E was coated onto Nunc-Immunoplates (Nalge Nunc, Denmark)

Electrophoresis and immunoblotting

SDS–PAGE and Western blotting were performed as described (Tacke et al., 2001); 10 μg of purified PERV/5° and 1 μg of purified viral or recombinant p15E was used as antigen per lane.

In vitro neutralization assays

Neutralization assays were performed on human kidney 293 cells. A cell suspension (100 μl) containing 4500 cells per well was seeded into 96-well microtiter plates and 2 days later, cells were infected with cell-free virus-containing supernatant of PERV/5°-infected 293 cells ($1 \times 10^{4.31}$ $TCID_{50}$/ml). Cells were split 1:3 after 3 days. After 6 days infected cells were detected by PCR using the forward primer 5′GTA CGT ACG TGG ATC CCT AAT CAC AGG ACC GCA ACA and the reverse primer 5′ACG TAC GTA CGA ATT CTC AGT TGA ACC ATC CTT AAA ACC from the *pol* region of PERV. For this cells were freeze-thawed three times and 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 for 3 h at 56°C. To inhibit proteinase K activity, the solution was subsequently incubated for 10 min at 95°C. In the inhibition experiments

recombinant p15E and peptides were dissolved in PBS and added at a concentration of 25 μg/well.

Mapping of epitopes

The entire amino acid sequence of p15E/PERV A was synthesized as a cellulose-adsorbed peptide spot library of 13-mer peptides, overlapping by 11 amino acids (Jerini Biotools) using a standard protocol (Kramer and Schneider-Mergener, 1998). The membrane was incubated with a 1:5000 immune serum solution (for control with preimmune serum) for 3 h, washed three times with Tris-bufferd saline, pH 7.5, with 0.05% Tween 20 (Sigma) for 15 min, and incubated for 2 h with peroxidase-conjugated secondary antibody diluted 1:10,000. Binding was detected using a chemiluminescence detection solution (ECL, Amersham Pharmacia Biotech).

Acknowledgments

This work was supported by the German Ministry of Health. We thank Dr. S. Norley for critical reading of the manuscript, Dr. C. Wilson for the generous gift of PERV-NIH/3°, and M. Lau and C. Schmidt for technical assistance.

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