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Generation of neutralising antibodies against porcine endogenous retroviruses (PERVs)

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ABSTRACT

Antibodies neutralising porcine endogenous retroviruses (PERVs) were induced in different animal species by immunisation with the transmembrane envelope protein p15E. These antibodies recognised epitopes, designated E1, in the fusion peptide proximal region (FPPR) of p15E, and E2 in the membrane proximal external region (MPER). E2 is localised in a position similar to that of an epitope in the transmembrane envelope protein gp41 of the human immunodeficiency virus-1 (HIV-1), recognised by the monoclonal antibody 4E10 that is broadly neutralising. To detect neutralising antibodies specific for PERV, a novel assay was developed, which is based on quantification of provirus integration by real-time PCR. In addition, for the first time, highly effective neutralising antibodies were obtained by immunisation with the surface envelope protein of PERV. These data indicate that neutralising antibodies can be induced by immunisation with both envelope proteins.

Introduction

Xenotransplantation using porcine cells or organs may offer a potential solution to the shortage of human donor organs. Prior to the clinical use of porcine xenotransplants, three main hurdles must be taken: immunologic rejection, physiological incompatibility, and the risk of transmission of porcine pathogens. Designated pathogen-free breeding of pigs can prevent transmission of most porcine microorganisms. However, this is not possible in the case of porcine endogenous retroviruses (PERVs), which are integrated in the genome of all pigs (Le Tissier et al., 1997), are released from normal pig tissues (Wilson et al., 1998; Martin et al., 1998; Tacke et al., 2000), and infect human cells at least *in vitro* (Patience et al., 1997; Specke et al., 2001a). Although different strategies aimed at preventing PERV transmission such as specific selection of animals expressing low amounts of PERV (Tacke et al., 2003) or generation of animals expressing PERV-specific siRNA (Dieckhoff et al., 2008; Ramsoondar et al., 2009) are under development, a preventive vaccine or passive immunisation of the recipient may be useful. We described the induction of neutralising antibodies by means of immunisation with the ectodomain of the transmembrane envelope protein p15E of PERV (Fiebig et al., 2003). Two epitopes were recognised by the serum—one, designated E1, in the fusion peptide proximal region (FPPR) of p15E, and another, E2, in the membrane proximal external region (MPER). Since there is no adequate animal model allowing analyses of the efficacy of PERV-specific neutralising antibodies *in vivo*, immunisations with p15E of the closely related feline leukaemia virus (FeLV) were performed in parallel. FeLV infects cats *in vivo* and causes leukaemia and immunodeficiency. When rats, goats and cats were immunised with the ectodomain of p15E of FeLV, neutralising antibodies were easily induced and a similar epitope pattern as in the case of PERV was observed (Langhammer et al., 2005, 2006). When cats immunised with p15 alone were challenged with infectious FeLV, protection from antigenemia was shown in 50% of the animals (Langhammer et al., 2011), whereas all animals immunised with the surface envelope protein gp70 alone or together with p15E were protected (unpublished data). In an additional study, rats were immunised with p15E of FeLV together with its recombinant surface envelope protein gp70 and higher titres of neutralising antibodies were obtained than in the case of immunisation with each antigen alone (Langhammer et al., 2010). Immunotherapy studies in mice infected with murine leukaemia virus (MuLV) showed a better therapeutic effect when animals were treated with antibodies against p15E and gp70 in combination (Schwarz et al., 1984). The investigation of successful vaccines against gammaretroviruses may have implications for HIV vaccine research because of the structural and functional similarity of all retroviral Env proteins. Interestingly, the epitopes found in the MPER of FeLV and PERV are localised in positions similar to that of the epitopes of 2F5 and 4E10, antibodies broadly neutralising HIV-1. Despite the great evolutionary distance, a limited sequence homology between the epitope sequences in the transmembrane envelope protein gp41 of HIV-1 and p15E of PERV

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was found (Fiebig et al., 2003). However, up to now all attempts to induce antibodies broadly neutralising HIV-1 have failed.

Here we confirm and extend our previous report on neutralising antibodies against PERV (Fiebig et al., 2003). We show generation of antibodies recognising the MPER of p15E in four animal species. The ability to induce such antibodies in different species indicates that similar antibodies may be also induced in other species including non-human primates and humans, if required. For the first time, neutralising antibodies specific for the surface envelope protein gp70 of PERV were induced. This allows performing immunisation studies with a combination of surface and transmembrane envelope proteins.

Results

Production and characterisation of antigens used for immunisation

In order to produce recombinant antigens for immunisation, the sequence of the ectodomain of p15E (amino acids 488–596, accession number HQ688786), and of the surface envelope protein gp70 (amino acids 49–487, accession number HQ688785) of PERV-A were amplified by PCR. cDNA from PERV-producing PK-15 cells was used as template. The fragments were cloned using the expression vectors pCal-n or pET22b(+) (Fig. 1). After expression in *Escherichia coli* BL21, p15E was purified by calmodulin binding protein and gp70 by His tag affinity chromatography. The recombinant p15E had a molecular mass of 12 kDa (Fig. 2A). Recombinant gp70 comprises 439 amino acids, the furin cleavage site is located at position 462, and the recombinant protein contains the first 25 amino acids of p15E in addition to gp70. Therefore this protein corresponds to the recombinant surface envelope protein p45 of FeLV used in the commercial vaccine Leucogen, which effectively protects cats from FeLV induced disease and antigenemia (Hofmann-Lehmann et al., 1995). At the N-terminus of the recombinant surface envelope protein of PERV, a pelB leader sequence is attached to direct the protein into the periplasma of *E. coli* producer cells. Constructs not containing this leader sequence did not show expression (data not shown). Since the pelB leader sequence was not cleaved, the recombinant surface envelope protein had a molecular mass of 54 kDa as shown by SDS-page analysis (Fig. 2A).

Induction and characterisation of binding antibody response

One goat (#62), 10 mice, 4 rats and two guinea pigs were immunised with the recombinant surface envelope protein gp70; three goats (#16, #346 and #355), 10 mice, 9 rats in the first experiment and 4 rats in the second, as well as two guinea pigs were immunised with the transmembrane envelope protein p15E. 10 mice, 4 rats and two guinea pigs were immunised with both, gp70 and p15E, and 10 mice, two guinea pigs and 4 rats were immunised with adjuvant alone. Goat #20 had been immunised previously with p15E (Fiebig et al., 2003). Specific antibodies were detected in the sera of all these animals. The sera reacted with the recombinant antigens used for immunisation (not shown) as well as with the viral protein in ELISAs and Western blot analyses (Fig. 2A). The titres of the binding antibodies as determined in ELISAs were in the range of 10^4 to 10^6, similar to the titre of the goat serum against p15E reported previously (Fiebig et al., 2003) (Fig. 2B). In parallel the responses against gp70 were analysed (Fig. 2C).

Measurement of neutralisation by provirus integration using a PCR

In first experiments, neutralisation was measured by provirus integration using a PERV-specific PCR (Fiebig et al., 2003). The neutralisation of PERV/5°, a high-titre recombinant PERV-A/C virus infecting human 293 cells (Denner et al., 2003), and PERV-B, also infecting human cells, was analysed. The viruses were titred in the absence and presence of the immune serum and the $TCID_{50}$ was determined. Whereas the $TCID_{50}$ of the virus in the absence of immune serum was $10^{4.31}$, it was reduced to up to 1% in the presence of neutralising sera (Supplementary Table 1), indicating 99% neutralisation at a serum dilution of 1:10. Two goat sera (#20, #16) and 9 rat sera were found to neutralise in this range. The sera did not cross-neutralise HIV-1, MuLV and FeLV (not shown).

Generation of a novel neutralisation assay based on measurement of provirus integration using a duplex real-time PCR

To improve detection of PERV-specific neutralising antibodies in the immune sera, a novel assay was established, which is based on quantification of the neutralising activity by measuring viral DNA in the cells using a real-time PCR. Neutralisation was measured by estimating the difference between provirus integration in control cells (virus treated with medium or preimmune sera) and provirus integration in cells incubated with virus treated with immune sera. For this assay, also human 293 cells and PERV/5°, a PERV-A/C recombinant virus, were used. This new neutralisation assay also allows detecting cytotoxic effects of the sera on target cells by measuring the cellular gene GAPDH in parallel to the PERV provirus. Cytotoxic effects that may lead to false-positive results may be expected when the copy number of GAPDH is lower than in control samples. Since primers and probe correspond to highly conserved regions in the *gag* sequence, PERV-A, PERV-B and PERV-C could be detected in the assay. This assay is universal, as all types of human cells that can be infected by PERV may be used. If cells are used that can be infected by PERV and by other retroviruses, e.g., HIV-1, virus-specificity of the immune response can be analysed. When the efficiency of the duplex PCR was analysed using serial dilutions of PERV and GAPDH in buffer or human DNA, the slopes of the regression curves indicated efficiencies of almost 100% (Fig. 3A, B). There was no interference between both real-time PCRs, and the absolute detection limit was 10 copies of PERV provirus in 25 µl reaction mix.

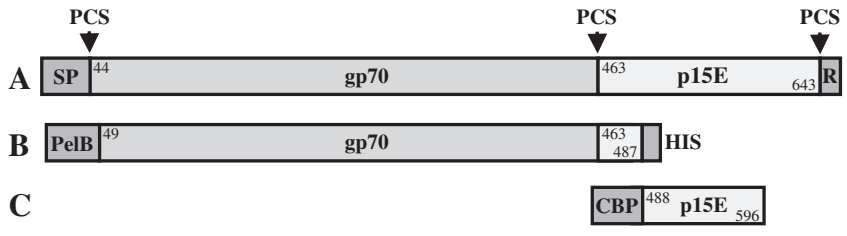

Fig. 1. Schematic presentation of the viral envelope proteins and antigens used for immunisation. (A) Precursor envelope protein of PERV-A (numbering according accession number AJ133817), protease cleavage sites (PCS) are marked with arrow heads, SP—signal peptide. R—R peptide. (B) Recombinant gp70 as expressed using vector pET22b(+) with an N-terminal pelB leader sequence promoting translocation to the periplasma and a C-terminal His-tag. (C) Recombinant p15E with N-terminal fused calmodulin binding protein (CBP) as expressed using the vector pCal-n.

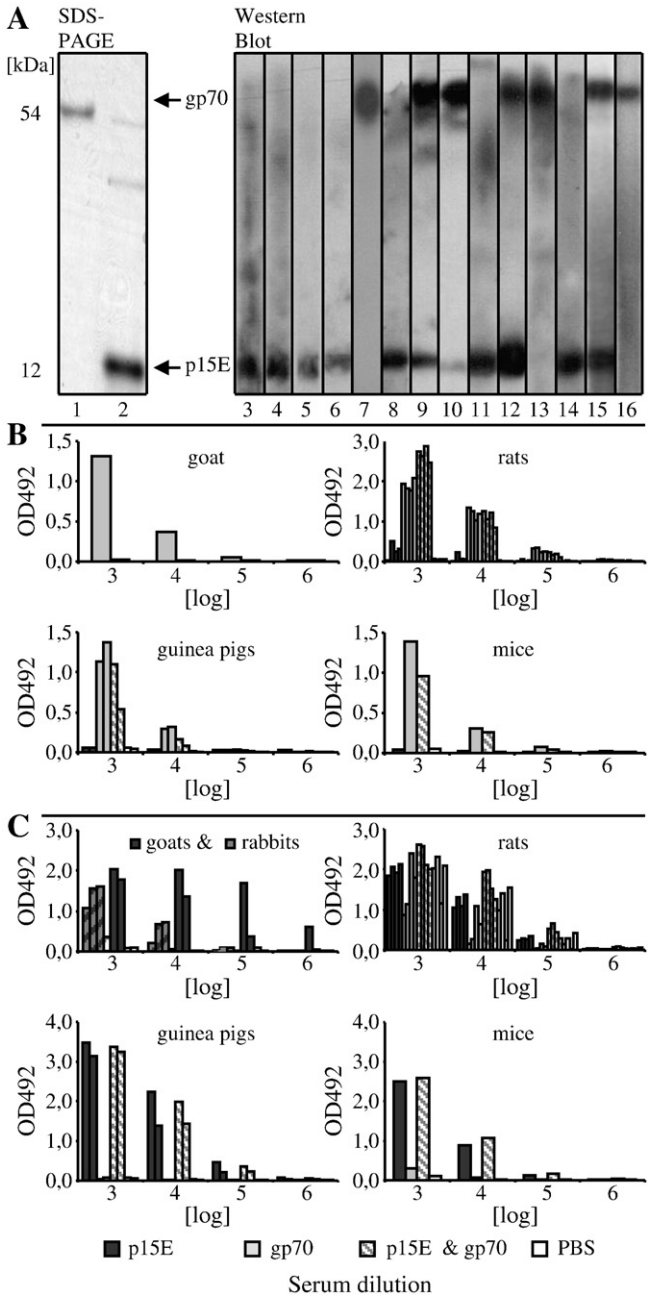

Fig. 2. (A) SDS-PAGE analysis of the recombinant antigens gp70 (54 kDa) and p15E (12 kDa) purified by affinity chromatography and Western blot analysis using purified virus particles as antigen and selected immune sera from different species (3–7, goats; 8–10, rats; 11–13, guinea pigs; 14–16, mice) immunised with gp70 (7, 10, 13, 16), p15E (3–6, 8, 11, 14) and a combination of gp70 and p15E (9, 12, 15). (B) Titres of antibodies binding to gp70 after immunisation of one goat (#62), 16 rats, 8 guinea pigs, and 40 mice (pooled serum) as estimated by ELISA using recombinant gp70 as antigen. (D) Titres of antibodies binding to p15E after immunisation of two goat (#346, #355), 3 rabbits, 16 rats, 8 guinea pigs, and 40 mice (pooled serum) as estimated by ELISA using recombinant p15E as antigen. Black columns indicate sera from animals immunised with p15E, gray with gp70, hatched with gp70 and p15E, and white with PBS.

Analysis of neutralising antibodies using the novel neutralisation assay

Sera from four goats immunised with p15E and the serum from the goat immunised with gp70, but not the preimmune sera, showed PERV-specific and dose dependent neutralisation (Fig. 3C). Purified immunoglobulins isolated from these sera were also neutralising, indicating that the neutralising activity was based on immunoglobulins (Fig. 3C). The titre was determined by setting 2ct values above the highest ct value of preimmune sera as cut off. The titre of goat serum 355 was 1:20, that of goat serum 346 1:40, that of the isolated immunoglobulins from serum 355 was 1:40 at 0,75 mg/ml and that of serum 62, specific for gp70, 1:320. Neutralising antibodies were also induced in a first experiment in 9 from 9 rats immunised with p15E (Supplementary Table 1). In a second experiment, 4 rats were immunised with p15E. However, in this experiment the preimmune sera showed binding antibodies against p15E (Fig. 2A, lane 10, Fig. 2C) and neutralising activity. In all cases the GAPDH-specific real-time PCR showed absence of cytotoxic activity in the sera. Unexpectedly, also the preimmune sera from guinea pigs and mice showed reduction of PERV infection. Since this unspecific activity could not be eliminated by purification of IgGs (data not shown), the neutralising activity of the immune sera from these mice, guinea pigs and rats in the second experiment could not be determined, despite the fact that this assay was successfully applied in the case of goats and rats in the first experiment.

Epitope mapping of the generated antibodies

To analyse the epitopes of the PERV-specific antibodies, an epitope mapping was performed using linear 15-mer peptides overlapping by 12 amino acids and corresponding to the entire p15E (Fig. 4A). Two main epitopes were identified when the serum from goat #16 was

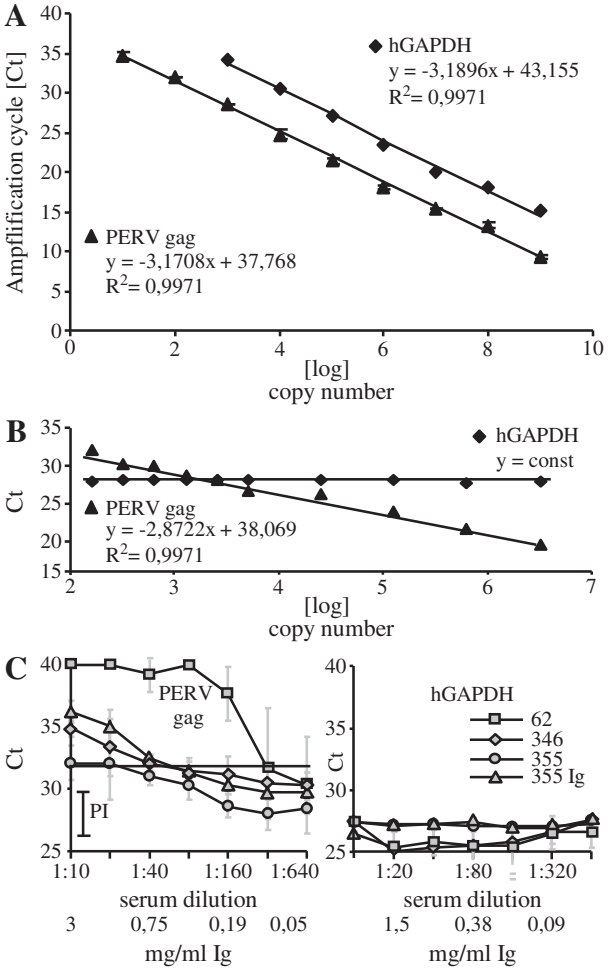

Fig. 3. Efficacies of the duplex real-time PCRs used in the neutralisation assay. Dilutions of PERV-A clone 220/14 (nearly 100% efficacy) and of an hGAPDH plasmid (nearly 100% efficacy) were performed in buffer (A) and human DNA (B) and the ct values were measured. (C) Neutralising activity of the goat serum 62, immunised with gp70, and goat sera 346 and 355, immunised with p15E. In addition, the neutralising activity of purified immunoglobulins from serum 355 was measured.

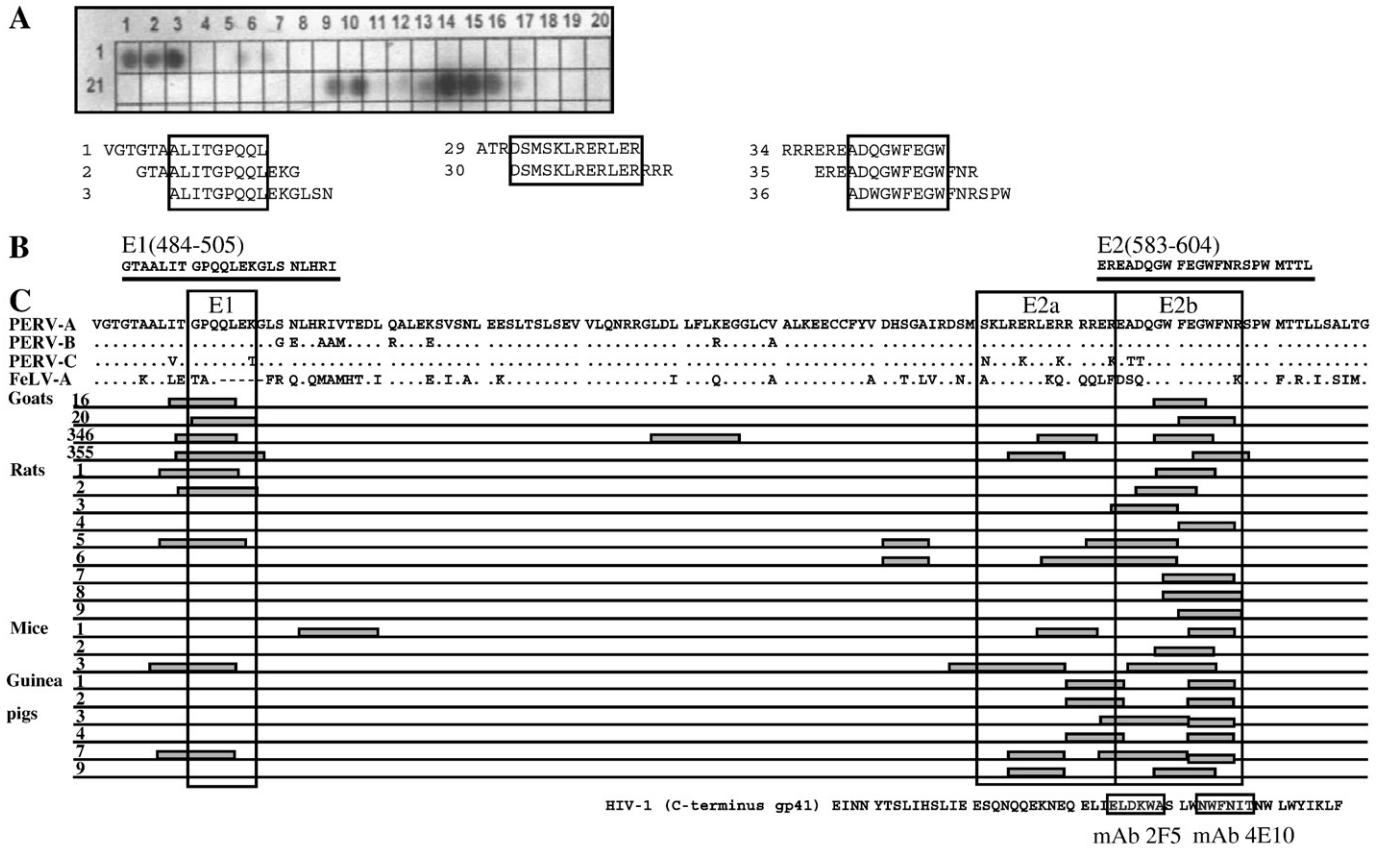

Fig. 4. (A) Example of an epitope mapping: serum from mouse #3, immunised with p15E. 15-mer peptides overlapping by 12 residues were fixed on the membrane, stained peptides were identified and epitopes determined. (B) Localisation and sequence of the peptides used for BIAcore analysis. (C) Summary of the epitopes recognised by each immune serum. All animals were immunised with recombinant p15E. The sequences of p15E of PERV-A, PERV-B, PERV-C and FeLV are shown and groups of epitopes were framed: E1 according to the epitope recognised by goat serum 20, E2a, E2b according to the epitopes identified in the case of FeLV (Langhammer et al., 2006). For comparison the sequence of gp41 of HIV-1 and the epitopes recognised by the monoclonal antibodies 2F5 and 4E10 are shown (Zwick et al., 2001). (D) Schematic presentation of the localisation of epitopes recognised by neutralising antibodies induced by immunising goats and rats with p15E of PERV, goats and rats with p15E of FeLV (Langhammer et al., 2005) and by monoclonal broadly neutralising antibodies isolated from HIV-infected individuals (Zwick et al., 2001). FP—fusion peptide, NHR—N-terminal helical region, CHR—C-terminal helical region, TM—transmembrane region.

analysed, confirming previous results with goat serum #20 (Fiebig et al., 2003); one epitope, E1, was located in the FPPR and the other, E2, in the MPER. All goat sera recognised the sequence GPQQL in the E1 region. Goat serum #346 showed an additional epitope localised in the immunosuppressive domain (Tacke et al., 2000). In two cases (goat sera #346 and #355), an arginine rich epitope (ERLERRR) was identified in the MPER (Fig. 4C). Whereas goat sera 16 and 20 recognised only one epitope in each domain, goat serum 346, 355 and 4 out of 9 rat sera recognised two epitopes, E2a and E2b, in the MPER. Most of the E2b epitopes contained the sequence FEGWFN. Whereas all rat, mice and guinea pig sera recognised epitopes in the MPER, only 5 of them recognised a sequence in the FPPR (Fig. 4C). Most importantly, the localisation of the epitopes is similar in the case of sera from goats and rats immunised with p15E of PERV and FeLV, the E2b epitope FEG**WFN** is identical in both viruses and corresponds in localisation and sequence to the epitope N**WFN**IT in gp41 of HIV-1 recognised by the broadly neutralising antibody 4E10 (Fig. 4C).

Specificity of the neutralising activity

To demonstrate the specificity of the neutralising activity, inhibition experiments were performed using recombinant gp70, and synthetic peptides corresponding to the p15E epitopes. When goat serum 62, specific for gp70, was incubated with recombinant gp70, a dose-dependent inhibition of the neutralisation was observed (Fig. 5). To investigate whether antibodies against both epitopes described for the goat sera immunised with p15E were involved in neutralisation, serum 355 was incubated with peptides corresponding

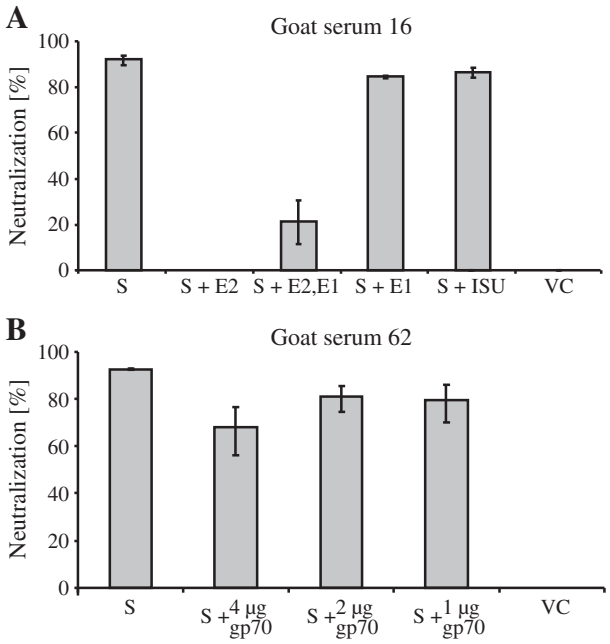

Fig. 5. Inhibition of the neutralising activity of (A) of goat serum #355 (S) obtained by immunisation with recombinant p15E by a peptide specific for the MPER (E2). In contrast, peptides corresponding to FPPR (E1) and to the immunosuppressive domain (ISU) were inactive in this assay (B) of goat serum #62 (S) obtained after immunisation with recombinant gp70 by different concentrations of gp70. VC-virus control without serum.

to the E1 and E2 epitopes as well as to the immunosuppressive domain as a control. Only the E2 peptide inhibited neutralisation significantly, indicating that the neutralising antibodies were directed mainly against the MPER of p15E. In the case of HIV-1 neutralising antibodies such as 2F5 and 4E10 also bind only to the MPER of gp41 (Zwick et al., 2001).

Surface plasmon resonance (SPR) analysis confirms antibody binding

To confirm binding of the sera to the epitopes identified by epitope mapping, a second method, SPR analysis using a BIAcore X100 was used. Two peptides, E1(484–505) GTAALIT**GPQQLE**KGLSNLHRI) and E2(583–604) (EREADQGW**FEGWFN**RSPWMTTL, epitopes recognised by goat serum 20 in bold) (Fig. 4B), and their randomised controls were used. SPR analysis showed that binding of the antibodies to the MPER peptide is typical of well-binding antibodies, such as the broadly neutralising antibody 2F5 to its epitope (Fiebig et al., 2009) (Fig. 6A). Whereas goat serum 355 binds nearly equally to E1(484–505) and E2(583–604) peptides, goat sera 16 (4.1 fold), 20 (3.6 fold) and 346 (1.7 fold) showed a stronger binding to the MPER-derived peptide E2 if compared to the FPPR-derived peptide E1 (Fig. 6B). Sera from four guinea pigs immunised with p15E alone (columns 1, 2) or in combination with gp70 (columns 3, 4) showed binding to the MPER-derived E2(583–604) in the BIAcore analysis. Sera of guinea pigs immunised with gp70 alone (column 5, 6) and control sera (column 7,8) showed as expected no reactivity specific for E1 or E2 (Fig. 6C). Sera from three out of four rats immunised with p15E and two out of four immunised with a mixture of p15E and gp70 showed binding to the MPER-derived peptide E2 (Fig. 6D).

Immune sera react with PERV on the cell surface

To elucidate the possible mechanism of neutralisation, the localisation of the epitopes recognised by the p15E-specific sera was analysed by immunofluorescence using non-permeabilised 293 cells infected with PERV/5°. Uninfected 293 cells were neither stained by the preimmune serum nor by the immune serum of goat 355 (Fig. 7A, B). In contrast, immune serum 355 reacted with larger virus accumulations located on the surface of PERV infected 293 cells (Fig. 7C, D). Such accumulation of PERV on the cell surface has been demonstrated by scanning electron microscopy (Karlas et al., 2010). Preimmune sera did not react with PERV producing 293 cells. These data indicate

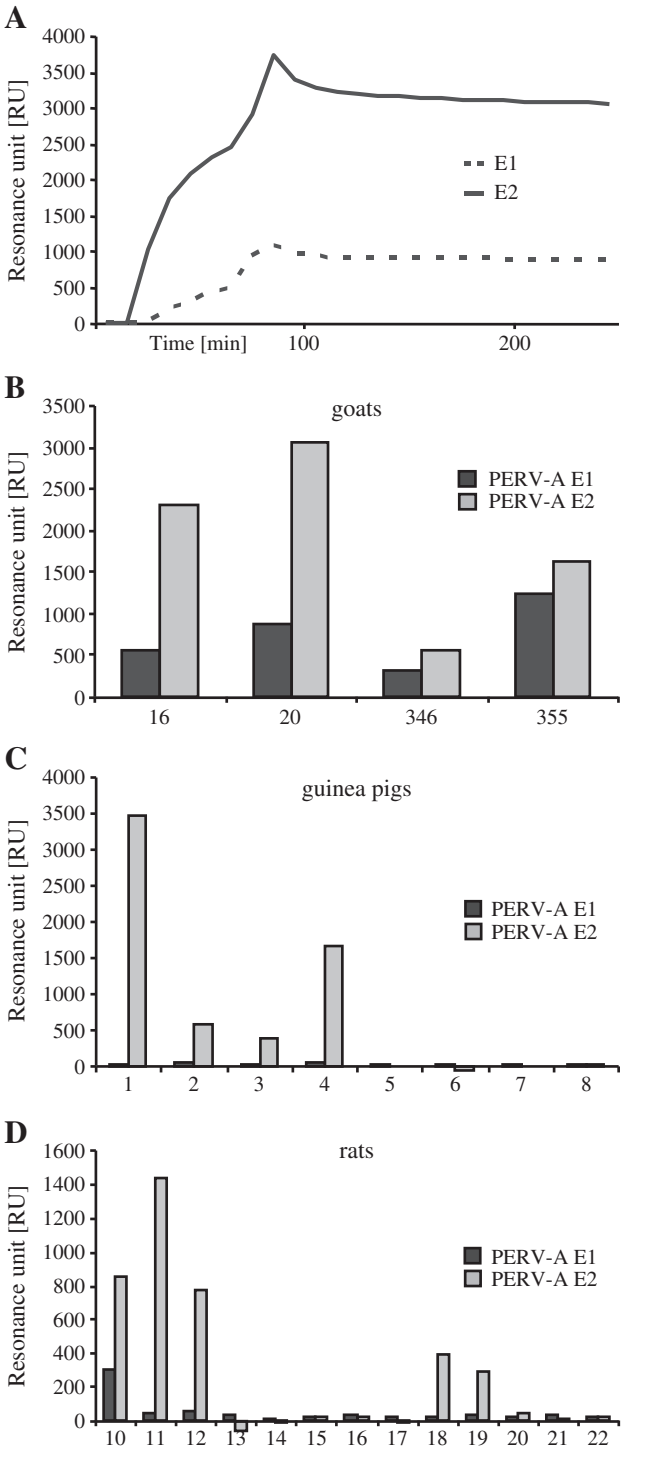

Fig. 6. SPR analysis of immune sera. (A) Binding curves of goat serum 20 to E1 and E2 peptides. (B) Binding of sera from four goats, (C) from eight guinea pigs, (D) and thirteen rats to the FPPR-derived peptide E1(484–505) (gray) and to the MPER-derived peptide E2(583–604) (black). Goats were immunised with p15E only, guinea pigs with p15E (1,2), p15E and gp70 (3,4), gp70 (5,6) and adjuvant alone (7,8). Rats were immunised with p15E (10–13), gp70 (14–17), p15E and gp70 (18–21) and adjuvant alone (22).

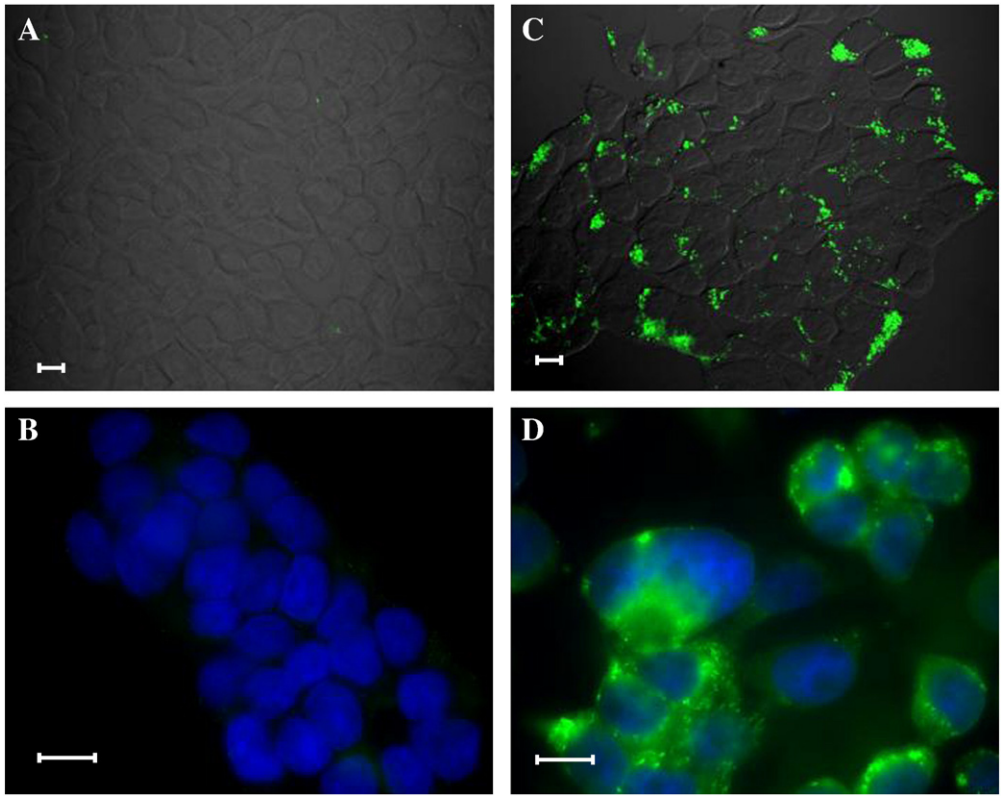

Fig. 7. Fluorescence microscopy showing absence of binding of preimmune serum with PERV infected 293 cells (A), and of neutralising goat serum 355 with uninfected cells (B), as well as binding of immune 355 serum with infected 293 cells (C, D); as secondary antibody an anti-goat FITC antibody was used. Immunofluorescence was analysed by confocal laser microscopy (A, C) and on an Axioskop (B, D).

that the epitopes in the virus protein p15E were exposed on the cell surface.

Discussion

Here we report for the first time induction of antibodies neutralising PERV by immunisation with the surface envelope proteins and extend our studies on induction of neutralising antibodies by immunising with the transmembrane envelope protein p15E. Most interestingly, one of the epitopes recognised by the anti-p15E sera induced in all species (FEG**WFN)** is localised in the MPER in a position similar to that of the monoclonal antibody 4E10 broadly neutralising HIV-1 in gp41 (N**WFN**IT, identical amino acids in bold). 4E10 broadly neutralises isolates of different clades of HIV-1 (Binley et al., 2004). Sera of all animals immunised with p15E of PERV recognised epitopes in the MPER as well as in the FPPR. Similar epitopes were found when goats, rats, mice, and cats had been immunised with p15E of FeLV (Langhammer et al., 2005, 2006) (Fig. 4D) as well as with p15E of the koala retrovirus (KoRV) (unpublished data).

According to a recent model of intramolecular conformational changes in the transmembrane envelope protein during virus infection (for review see Melikyan, 2008), it is very likely that E1 in the FPPR and E2 in the MPER are in close proximity after the interaction of the N-terminal and the C-terminal helical regions. Using SPR analyses, an interaction between peptides corresponding to E1 and E2 of gp41 of HIV-1 had been demonstrated, and the binding of the antibody 2F5 to its epitope in the MPER increased in the presence of a peptide derived from the FPPR (Fiebig et al., 2009). However, attempts to design an antigen based on gp41 able to induce neutralising antibodies failed until now since its conformation is unknown.

Using a novel neutralisation assay based on real-time measurement of proviral DNA we successfully demonstrated neutralising activity in the sera from goats and rats in the first experiment. Similar neutralisation assays were developed and applied for HIV-1 (Fiebig et al., 2009) and HIV-2 (Behrendt et al., 2009). Since there were neutralising activities in the preimmune and control sera from mice, guinea pigs, and rats in the second experiment, we were unable to detect neutralising antibodies in these sera. However, the identical localisation of the epitopes recognised by these sera and the similarity in the epitope sequence (Fig. 4C) suggest that the antibodies induced in these species are neutralising. False-positive neutralising activity in preimmune and control sera are common and seriously hamper vaccine development (Denner, 2011).

There is an urgent need to develop antiretroviral vaccines, first of all against HIV-1 and HIV-2 to stop the AIDS pandemic. There is also a need for a vaccine against human T-lymphotropic leukaemia viruses (HTLV-1 and -2). Recently, a new gammaretrovirus, XMRV, was described in patients with prostate carcinoma and chronic fatigue syndrome (CFS) and in some healthy individuals in the USA, but not in Europe (for review see Denner, 2010). If this virus is indeed distributed in the North American population, a vaccine may be required to eliminate the virus. A vaccine against PERV will be recommendable in case PERV is transmitted after xenotransplantation and infects humans *in vivo*.

Although numerous strategies are under development to prevent PERV transmission, such as inhibition of PERV expression by RNA interference using transgenic animals expressing PERV-specific siRNA (Dieckhoff et al., 2008; Ramsoondar et al., 2009), treatment with antiretroviral drugs such as azidothymidine (Qari et al., 2001; Shi et al., 2007; Stephan et al., 2001), a vaccine based on neutralising antibodies could be useful. These different approaches are aimed to increase virus safety, but their efficacy and impact on the safety of xenotransplantation remains to be validated.

Xenotransplantation of porcine cells or organs is a technology that may be available soon. First clinical trials using insulin producing pig islet cells are in progress. Worldwide more than 200 patients have

been treated with pig islet cells or *ex vivo* with spleen or liver cells (Paradis et al., 1999). Although transmission of PERV was neither observed in these first clinical trials (Elliott et al., 2000; Heneine et al., 1998; Valdes-Gonzalez et al., 2010), nor in animal models of xenotransplantation (Winkler et al., 2005; Moscoso et al., 2005; Switzer et al., 2001), the risk of a xenosis can by no means be excluded. PERV inoculation experiments in small animals and non-human primates showed either absence of virus transmission (Specke et al., 2009, 2001b, 2002), or false-positive transmission due to pseudotyping of PERV with murine retroviruses in mice (Deng et al., 2000; van der Laan et al., 2000; Clémenceau et al., 2002; Martina et al., 2005) or transient expression at a very low level (Argaw et al., 2004; Popp et al., 2007). However, when mice which do not have a receptor for PERV (Ericsson et al., 2003) were inoculated with cell-free virus, no transmission was observed (Irgang et al., 2005). A detailed and comprehensive overview of all clinical and preclinical xenotransplantation studies as well as of the PERV inoculation experiments are given in (Denner et al., 2009) These data indicate absence of productive replication *in vivo* despite infection *in vitro,* in cell lines of different species.

Methods

Cloning and purification of p15E and gp70

Two antigens were used for immunisation, (i) the ectodomain of p15E of PERV-A, expressed and purified as described (amino acids 488–596, accession number HQ688786) (Fiebig et al., 2003), and (ii) a recombinant protein corresponding to gp70 of PERV-A (amino acids 49–487, accession number HQ688785). The sequence was amplified using RNA from PK15 cells and the primers gp70-for-EcoRI and gp70-rev-SalI 5' (Table 1). The cloned sequence resembles the sequence of gp70 of FeLV, used in Leucogen for vaccination (Marciani et al., 1991). It was cloned into the pET-22b(+) expression vector (Novagen, San Diego, CA). Protein was expressed in *E. coli* BL21-CodonPlus(DE3)-RP (Stratagene, Amsterdam). Synthesis of the hexahistidine-tagged protein was induced with 0.05 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37 °C in LB-medium at OD_{600} of 0.7. After 3 hours bacteria were harvested by centrifugation at 8000*g* and disrupted by sonication. The insoluble recombinant protein was pelleted by centrifugation at 25,000*g* for 30 min, the pellet was resolved in 6 M GuHCl buffer, purified by metal chelating affinity chromatography using Ni-NTA (Qiagen), and the protein was dialysed against PBS and characterised by SDS-PAGE and Western blotting.

Immunisation, antisera and purification of immunoglobulins

Goats were immunised with 500 µg antigen, Wistar rats with 150 µg, Balb/c mice with 50 µg, rabbits with 250 µg, 340 µg or 170 µg and guinea pigs (rats, mice and rabbits were obtained from Charles River) with 200 µg p15E, gp70 or a mixture of both proteins intramuscularly and subcutaneously. Antigens were emulsified in complete Freund's adjuvant. The immune response was boosted by second and third immunisations after periods of 2 and 5 weeks using incomplete Freund's adjuvant. Immunoglobulins were purified using 2 M ammonium sulphate; IgGs were purified using the Ab SpinTrap™ kit (GE Healthcare) as recommended by the manufacturer.

Peptides

Biotinylated peptides E1(484–505) GTAALITGPQQLEKGLSNLHRI, E2(583–604) EREADQGWFEGWFNRSPWMTTL (Fig. 4B), E1 randomised ALRLGQELISGHNAPIKTQTLG and E2 randomised TWPGEFWRSTNMLDWEQARFGE were synthesised by Genaxxon BioScience GmbH, Biberach, Germany. For the inhibition experiments modified E1 and E2 peptides (AALITGPQQLEKGLSNLHRICKKK; KKKCCREREADQGWFEGWFNRSPWM) as well as a peptide (VVLQNRRGLDLLFLKEGGL) corresponding to the immunosuppressive domain, synthesised by Jerini, Berlin, Germany, were used.

Western blot, ELISA

Western blot and ELISA were performed as described before (Tacke et al., 2001) using the recombinant proteins gp70 and p15E, or lysates of purified PERV preparations, respectively. In ELISA 0.5 µg/well recombinant proteins and in Western blot assays 1 µg of virus preparation was used; sera were diluted 1:100 to 1:250. A secondary antibody labelled with HRP was used for ECL detection.

Epitope mapping

The entire p15E of PERV (130 amino acids) was synthesised as a cellulose-adsorbed peptide spot library of 15-mer peptides overlapping by 12 amino acids (JPT Peptide Technologies, Germany) using a standard protocol of the supplier. Sera were diluted 1:1000 and binding was detected using a chemiluminescence detection solution (ECL, Amersham Pharmacia Biotech).

Real-time PCR

For quantification of PERV proviral DNA the primers gag-for and gag-rev located in the *gag* gene and a specific PERV-*gag* probe (Table 1) were used in a duplex real-time PCR. The reference gene GAPDH was amplified with the primers GAPDH-for and GAPDH-rev and quantified using an hGAPDH-probe. The 22 µl reaction mixture consisted of 1× PCR buffer with 1.5 mM $MgCl_2$, 0.5 µM each of dATP, dCTP, dGTP, dTTP, 5 pmol of each primer, 5 pmol of probe, 1.25 U AmpliTaq Gold® polymerase and 3 µl lysate. The thermal cycling conditions used were 10 minutes at 95 °C followed by 50 cycles of 1 minute at 95 °C, 1 minute at 59 °C and 30 seconds at 72 °C in a Stratagene MX4000 machine. Efficacy was calculated by measuring ten fold serial dilutions of a subcloned hGAPDH PCR-fragment and the molecular clone PERV-A 14/220, kindly provided by Y. Takeuchi.

Table 1
Primers and probes.

Primer/probe	Sequence 5′–3′	Direction	Location	Accession no.
hGAPDH-for	GGCGATGCTGGCGCTGAGTAC	+	365..385	AF261085
hGAPDH-rev	TGGTCCACACCCATGACGA	−	495..513	AF261085
hGAPDH-probe	HEX-TTCACCACCATGGAGAAGGCTGGG-BHQI	+	407..430	AF261085
PERV-gag-for	TCCAGGGCTCATAATTTGTC	+	1213..1232	AJ293656
PERV-gag-rev	TGATGGCCATCCAACATCGA	−	1289..1308	AJ293656
PERV-gag-probe	FAM-AGAAGGGACCTTGGCAGACTTTCT-BHQ1	+	1244..1267	AJ293656
Gp70-for-EcoRI	AGAATTCG-AGCCCGAACTCCCATAAACCC	+	6329..6349	AJ293656
Gp70-rev-SalI	AAAGTCGAC-GGCAGCCGTTCCTGTTCCC	−	7627..7645	AJ293656

Neutralisation assays

Two types of neutralisation assays were performed using virus-containing cell-free supernatants produced by human embryonic kidney 293 cells infected with PERV/5°. This virus is a PERV-A/C recombinant repeatedly passaged on human cells which was associated with elevated titres and genetic alterations in its long terminal repeats (LTR) (Denner et al., 2003). In addition, PERV-B kindly provided by D. Onion, was also used. 100 µl uninfected 293 cell (1.5×10^5/ml) were seeded in 96 well plates and incubated for 4 hours at 37 °C in 5% (v/v) CO_2. Sera were decomplemented by heat inactivation (30 min at 56 °C) and 20 µl were mixed with 80 µl of a PERV dilution, incubated for 30 min at 37 °C and added to the 293 cells. Virus dilutions resulting in stable Ct values (25 to 27) were considered as optimal for the neutralisation assay. After incubation for 72 h at 37 °C cells were examined by light microscopy for viability and the medium was removed. Cells were lysed by heating at 95 °C for 30 min, freezing at −20 °C for 6 hrs, and incubation with lysis buffer (nuclease free water containing 0.2 mg/ml proteinase K and 10% (v/v) 10×PCR-buffer) at 60 °C for at least 3 hrs. Proteinase K was heat inactivated (30 min at 95 °C). In the first assay proviral DNA was measured by a simple PCR, virus was diluted in the absence and presence of a dilution of the immune serum of 1:10 and the $TCDI_{50}$ was determined. In the second assay proviral DNA was quantified by real-time PCR (see below), 3 µl of the lysate were used in the duplex real-time PCR. To inhibit neutralisation, recombinant gp70 or synthetic peptides were diluted and incubated with immune serum for 20 min and both with the virus another 20 min.

Calculation of neutralisation efficacy

In the assay based on real-time PCR, neutralisation was defined as reduction of provirus integration in the presence of immune serum. The ct values of GAPDH were identical in all samples, indicating absence of toxic effects of the sera. In Fig. 3 neutralisation was expressed as increase of PERV specific ct values (the higher the ct value in the real-time PCR the lower the provirus load). In Fig. 5 percent of neutralisation was calculated as (ct value of PERV − ct value of GAPDH) in the presence of serum − (ct value of PERV − ct value of GAPDH) in the absence of serum (ΔΔct) and the $2^{\Delta\Delta Ct}$method (Livak and Schmittgen, 2001), % neutralisation (NT) was calculated as $NT = 100–100/2^{\Delta\Delta Ct}$. A ΔCt of at least 2, which corresponds to a provirus reduction of 75%, was defined as a significant difference in provirus integration.

Surface plasmon resonance (SPR) analysis

SPR analysis was performed using a BIAcore X100 (GE Healthcare, Waukesha, Wisconsin, USA). Two streptavidin (SA) chips (GE Healthcare) were generated. The first chip was coated on flow cell (Fc) 2 with 1200 resonance units (RU) of the biotinylated sequence E1(484–505), and on Fc 1 with 1200 RU of the biotinylated E1-randomised peptide. Fc 2 of the second chip was coated with E2(583–604) and Fc 1 with the randomised E2 peptide, respectively. The immune sera were tested undiluted. As running buffer 1× HBS-EP + buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0,05% Surfactant P20, pH 7,4; GE Healthcare) containing 1 M urea was used. The binding time for the sera to its ligand was 60 sec and the dissociation time 180 or 240 sec. For the regeneration of the chips 50 mM glycine/HCl, pH 1.5 (in the case of guinea pig sera pH 2.0) was used. All binding analyses were performed at 25 °C at a flow rate of 10 µl/min.

Immunofluorescence

PERV/5° producing and uninfected 293 cells were grown on culture dishes with a hydrophobic membrane (Petriperm, Heraeus), washed with PBS and fixed with 2% paraformaldehyde for 2 h. After washing with PBS, the membrane was cut in small pieces. Cells were blocked with PBS supplemented with 0.1% gelatine and 1% rabbit serum. Preimmune and serum of goat 355 immunised with p15E were tested at a dilution of 1:500. After washing the cells were incubated with FITC-labelled rabbit anti-goat IgG (Sigma) at a dilution of 1:160. Finally, cells were embedded in Moviol 4-88 (Roth) and 0.1 µg/ml Bisbenzimide H 33258 (Sigma) solution, and the surface fluorescence was analysed by fluorescence microscopy (Axioskop2, Axiovision Rel. LE 4.5 and CLSM Mikroskop 510 META, Carl Zeiss AG Oberkochen, Germany)

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