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Productive infection of a mink cell line with porcine endogenous retroviruses (PERVs) but lack of transmission to minks in vivo

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Summary. Porcine endogenous retroviruses (PERVs) are considered a special risk for xenotransplantation because they are an integral part of the porcine genome and are able to infect cells of numerous species including humans in vitro. Among these cells, the mink lung epithelial cell line Mv1Lu could be productively infected with PERV. Provirus integration was detected by PCR, expression of viral proteins was shown by immunostaining and reverse transcriptase was detected in cell supernatants. PERV produced from mink cells could infect both, uninfected mink Mv1Lu cells and uninfected human 293 cells, with considerably higher virus production by human cells. Typical type C retroviruses were observed in PERV-infected mink cells using electron microscopy together with numerous multivesicular body (MVB)-like structures containing small virus-like particles, not present in uninfected mink cells. These MVBs could be stained with PERV-specific serum. In an attempt to establish a small animal model, PERV grown on mink cells was inoculated into adult and newborn American minks. Neither antibody production against PERV nor integration of viral DNA or production of viral proteins in tissues of different organs could be detected 12 weeks post virus inoculation, indicating that PERV infection had not occurred.

Introduction

To respond to the increasing shortage of organs for allotransplantation, alternative methods are continuously being developed. One of the most promising approaches at present is xenotransplantation using pig cells, tissues or organs. In addition to the economical and ethical reasons, pigs are favoured as organ donors because of their low rate of infection with microorganisms compared to non-human primates [1]. While most known pathogens can be eliminated by breeding, treatment

and containment, porcine endogenous retroviruses (PERVs) cannot, because of their presence in the genome of all pig strains [9, 21]. At least two subtypes of PERV, PERV-A and PERV-B, are able to infect human cells in vitro [13, 14, 16, 20, 25, 32, 33]. For these reasons PERVs represent the greatest potential risk in the context of xenotransplantation [8]. However, studies of human recipients who have had short term contact with porcine cells and tissues [19, 29] as well as initial transplantations of porcine cells to baboons [15, 28] showed no evidence of PERV transmission. Furthermore, attempts to establish small animal models with naive and immunosuppressed rats and guinea pigs did not result in infection [26]. However, infection of SCID mice with PERV was recently reported [5, 12], indicating that trans-species transmission of PERV in vivo is generally possible. In order to investigate whether minks could be infected with PERV in vivo, adult animals were inoculated with virus grown on and adapted to mink cells. Due to the incorporation of cellular proteins while budding on the host cells' membrane, PERV replicating on mink cells should be adapted in a way which enables them to a better escape of immune responses against cellular antigens in the viral membrane. In addition to adult minks, newborn animals were also inoculated with mink-adapted PERV as their underdeveloped immune system partially simulates pharmacological immunosuppression. Neither antibodies against PERV nor proviral DNA was detected in any animal, indicating that infection had not occurred.

Materials and methods

PERV producing cell lines

For the in vitro infection of mink cells PERV produced from the infected human kidney cell line 293 (32; kindly provided from Prof. Dr. R. Weiss, Wohl Virion Centre, Windeyer Institute of Medical Sciences; London, UK) was used. For the in vivo infection of animals, PERV produced from the mink lung epithelial cell line Mv1Lu (ATCC-Nr.: CCL-64, USA) was used [26].

Cell lines used for in vitro infection

As target cells both the human kidney cell line 293 (from C. Wilson, FDA, Washington, USA) and the mink lung epithelial cell line Mv1Lu were used. Cells were incubated overnight with cell free supernatant of cultured PERV-producing cell lines in the presence of 8 μg/ml polybrene (Sigma, Deisenhofen, Germany).

Infections of animals

For the in vivo infection experiment 3 adult and 3 newborn American minks (*Mustela vison*, Artemis, Melle, Germany) were used. The animals were housed in single cages, except the newborn minks which remained with their mothers. These two mothers were not inoculated with PERV but used as negative control animals in the experiment. Supernatants from PERV-producing Mv1Lu cells were collected, cell debris was removed by centrifugation at $3500 \times g$ for 10 min and 10000 rpm (SW 28, Beckmann) and virus was pelleted by ultracentrifugation (3 h at 28000 rpm, SW 28, Beckmann). Pelleted virus was titred on uninfected Mv1Lu cells by PCR. Concentrated virus was resuspended in 1 ml PBS and inoculated intraperitoneally (i.p.). The newborn animals were inoculated in the same manner one day after birth. Blood

was taken by cardiac puncture every four weeks after virus inoculation for processing to serum. Animals were euthanised 12 weeks post infection, blood and organs were removed and used for DNA isolation and immunohistology.

DNA-isolation

DNA from cultured cells and heparinised blood was isolated using the DNA blood mini kit from Qiagen (Hilden, Germany). DNA from organs was isolated using the Qiagen DNeasy tissue kit (Hilden, Germany).

Titration of PERV

1×10^6 uninfected Mv1Lu cells per well were seeded in 8 replicates in a 96 well plate and incubated for 12 h with cell-free pelleted virus produced from infected Mv1Lu cells in the presence of 8 μg/ml polybrene. Cells were cultivated for 3 weeks and lysed for 3 h at 56 °C in a lysis reagent containing 20 mg/ml proteinase K (Life Technologies) and PCR-buffer (50 mM KCL; 1.5 mM $MgCl_2$; 10 mM Tris-HCL; pH 8.4; Applied Biosystems). The enzyme was heat-inactivated by incubation at 95 °C for 10 min. Four μl of this crude extract were used as template in a nested PCR as described later on.

PCR

PCR was performed as described [25, 26] using primers specific for PERV *gag* [19], for *env* of PERV-A and PERV-B [13] and for PERV *pol* [2]. Three μl of the first amplification using PERV *pol* primers were used as template in a nested PCR employing additional PERV *pol* primers [2]. Amplification was performed using a Biozym cycler (Oldendorf, Germany) and standard conditions with one initial cycle of 95 °C for 10 min, 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min for 35 cycles followed by an extension reaction at 72 °C for 10 min.

Sera

For immunological detection assays PERV-specific antisera were generated using recombinant transmembrane protein p15E and purified viral p27Gag [27, 29, 31].

Immunoperoxidase assay (IPA)

IPA was performed as described previously [26, 27]. Cells were trypsinised and seeded in six-well plates (1×10^5 cells / well) coated with poly-D-lysine (Greiner, Frickenhausen, Germany). After incubation for 4 h at 37 °C, 5% CO_2 and 98% humidity, cells were washed twice with PBS and fixed with methanol overnight at −20 °C. Cells were treated with 2% fat free milk powder (Marvel, UK) in PBS for 1 h to block unspecific antibody binding. After blocking, the cells were incubated for 1 h with antiserum specific for recombinant PERV p15E or purified p27Gag (diluted 1:100 in blocking solution). After washing four times with PBS, protein G labelled with horseradish peroxidase (1:5000 in blocking solution) was added to the cells. After 1 h incubation, cells were washed again four times with PBS and the substrate H_2O_2 and the chromogen 3-amino-9-ethylcarbazole (AEC, Sigma, Deisenhofen, Germany) was added. Photomicrographs were taken using a Zeiss Axiovert inverse light microscope.

Immunofluorescence

Cells were seeded on glass slides and fixed with 2% paraformaldehyde (Merck, Darmstadt, Germany) for 1 h at room temperature. After washing twice in PBS-Dulbecco, membranes

were permeabilised with 0.5% Triton X-100 (Sigma, Germany) for 15 min at room temperature. After washing again three times in PBS-Dulbecco cells were blocked with 1% non fat dry milk for 30 min at room temperature. Thereafter cells were incubated with sera specific for recombinant PERV p15E or purified viral p27Gag (diluted 1:100 in blocking solution) for 1 h at 37 °C. Cells were washed three times in PBS-Dulbecco and incubated with species-specific FITC-labelled anti-IgG antibody (Dianova; diluted 1: 200 in blocking solution) for further 30 min at room temperature. Finally, cells were washed twice in PBS-Dulbecco and covered with moviol (Calbiochem, La Jolla, USA). Photomicrographs were taken using a Zeiss Axiophot fluorescence microscope.

Immunohistology

Mink tissue samples were fixed in concentrated methanol (70% v/v) for 24 h, embedded in paraffin and slides were prepared according to standard techniques. Subsequently, slides were de-paraffinised and endogenous peroxidase was inactivated with 3% H_2O_2. Blocking was carried out using 1% bovine serum albumin (BSA). PERV antigens were detected using a PERV p15E-specific goat serum [27] diluted between 1:10 and 1:320 and a peroxidase-conjugated rabbit anti-goat-IgG monoclonal second antibody (Sigma, Heidelberg, working solution 1:5000). Sections were subsequently stained with H_2O_2 and diaminobenzidine (DAB, Sigma, Heidelberg).

Electron microscopy

Cells were fixed at room temperature for 45 min using a freshly prepared 2.5% solution of glutaraldehyde in warm medium. Fixed cells were scraped off the culture plate, resuspended in warm liquid agarose and immediately chilled on ice. After cutting the agarose into small cubes, cells were post-fixed in 1% OsO_4 in PBS, dehydrated in a graded series of ethanol and embedded in Epon 812 according to standard protocols. Polymerised Epon blocs were cut into 80 nm sections on a Leica Ultracut 4 microtome and contrasted with 2% uranyl acetate for 10 min and 2% lead citrate for 2 min at room temperature. Micrographs were taken on a Zeiss CEM 902 electron microscope using ESI mode.

Western blot

Western blot analysis was performed as described previously [30, 31]. Supernatants from PERV-producing 293 cells were collected and cell debris was removed by centrifugation at 10000 × g for 30 min. Virus was concentrated by ultracentrifugation (54.000 × g for 3 h) resuspended and loaded onto a sucrose gradient (20–50%, 200.000 × g for 3 h). Fractions were collected after centrifugation and subjected to denaturing 10% SDS-PAGE using tricine buffer and transferred to PVDF-membranes by semi dry-blotting. Membranes were blocked using 0.1% Tween 20 and 1% BSA in Tris-buffered saline (TBS), incubated with 1:100 dilutions of sera for 12 h at 4 °C and, after washing, incubated with a 1:5000 dilution of protein G labelled horseradish peroxidase for 2 h at room temperature. Antibody binding was visualised using metal-enhanced diaminobenzidine (Pierce) and peroxide. As positive controls, goat and rabbit antisera raised against purified PERV particles, purified viral proteins, and purified recombinant viral proteins were used [30, 31].

Measurement of reverse transcriptase (RT) activity

For detection of RT activity, a commercial assay specific for type C retroviruses with Mn2+ preference was used (CavidiTech, Uppsala, Sweden) and measured on a Tecan Spectra 4 ELISA-reader at 405 nm.

Results

Productive infection of the mink cell line Mv1Lu

After inoculation of the mink cell line Mv1Lu with cell-free supernatants from PERV-producing human 293 kidney cells, proviral DNA was detected by PCR using different primers (Fig. 1). PERV-producing human 293 cells release

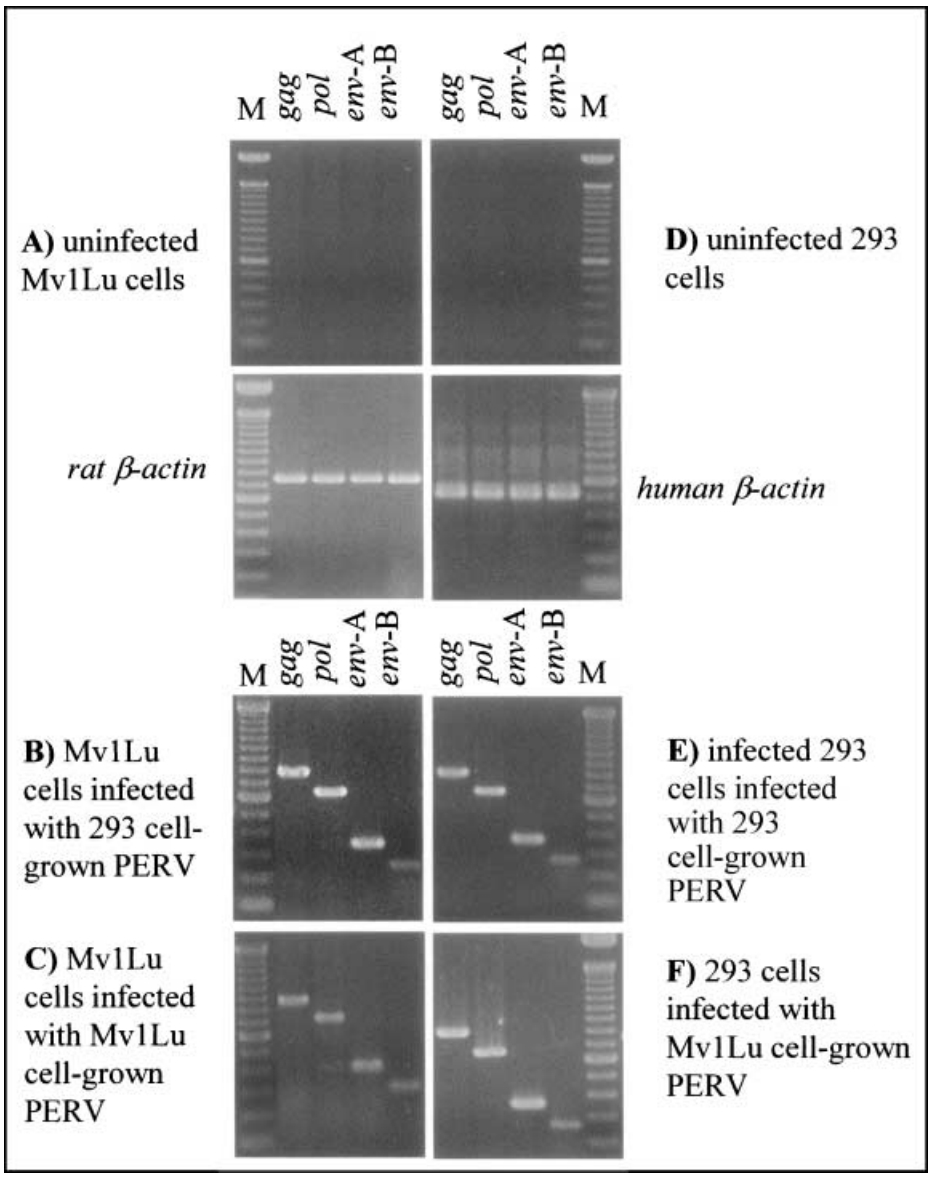

Fig. 1. PCR with DNA from uninfected and infected mink lung epithelial Mv1Lu cells (left) and human kidney 293 cells (right). The used primers were aligned from sequences of the *gag*, *pol* and *env* gene of PERV. To discriminate the subtypes of PERV, *env*-primers specific for PERV-A and PERV-B were used. To guarantee the quality of the DNA primers for β-actin were used. *M* 100 bp-ladder

a mixture of two polytropic subtypes of PERV, PERV-A and PERV-B, and both were shown to be transmitted to mink cells (Fig. 1). Furthermore, virus protein production was shown in infected cells, but not in uninfected control cells by immunoperoxidase assay (IPA) (Fig. 2) and immunofluorescence microscopy (see below). Reverse transcriptase (RT) activity was found in the

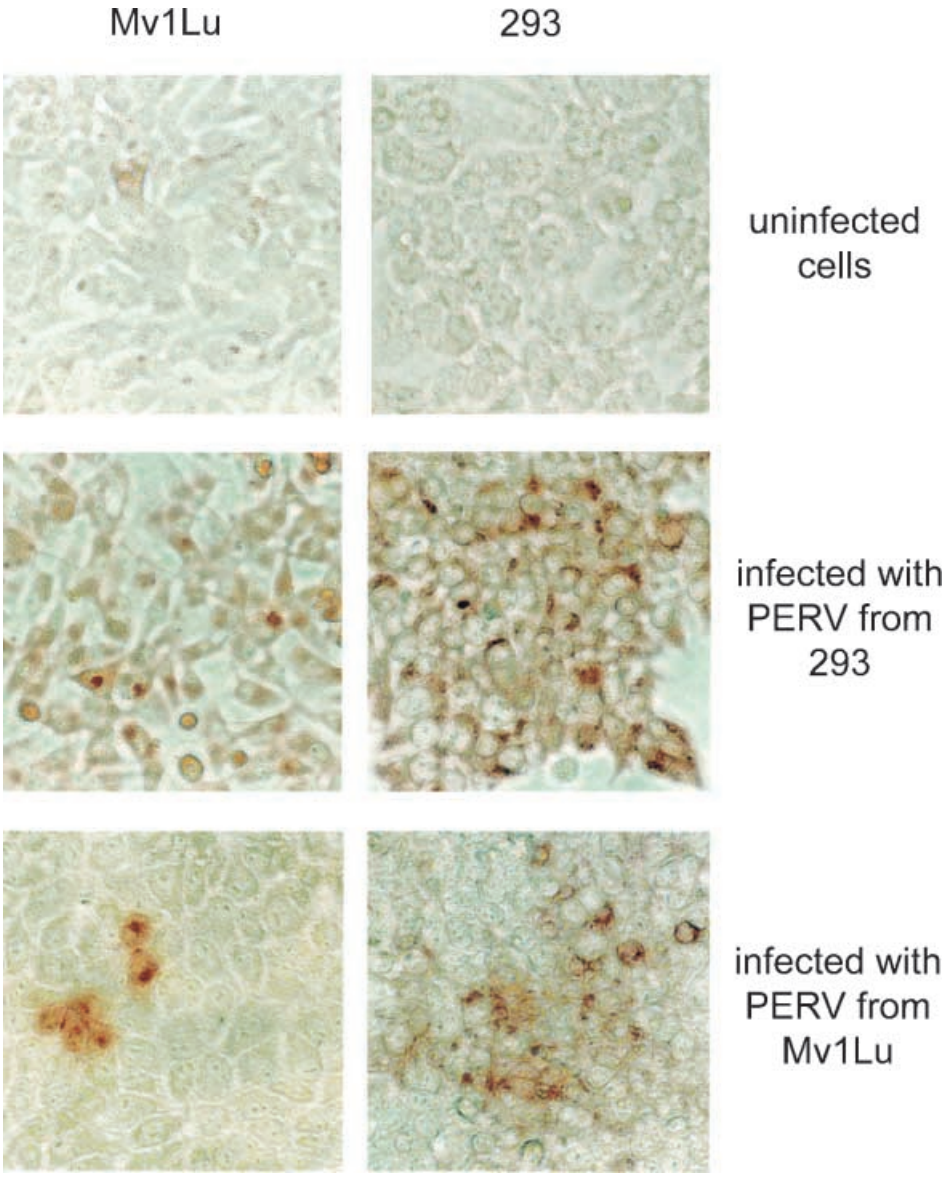

Fig. 2. Immunoperoxidase assay with uninfected and infected mink lung epithelial Mv1Lu cells (left) and human kidney 293 cells (right). Infection was monitored by showing expression of viral p15E visualized by coloring infected cells red. Microphotographs were taken using a Zeiss Axiovert inverse light microscope at a magnification of 100×

Table 1. RT assay using cell free supernatants of PERV infected mink and human cells

Cell line	Released RT activity [mU/ml]
Mv1Lu cells infected with 293 cell-grown PERV	$39 \pm 1{,}8$
Mv1Lu cells infected with Mv1Lu cell-grown PERV	$13 \pm 0{,}3$
293 cells infected with Mv1Lu cell-grown PERV	$37 \pm 2{,}1$

RT was produced by 1×10^7 cells in 20 ml medium in 3 days. Average and standard deviation is given for triplicates

supernatant of the infected mink cells (Table 1), indicating a productive PERV infection.

Transmission of mink cell-derived PERV to mink and human cells

Virus taken from cell-free supernatants of the PERV-infected mink cell line Mv1Lu was able to further infect Mv1Lu cells in a productive manner (Fig. 1, Fig. 2, Table 1). PERV from infected Mv1Lu cells was pelleted and titrated on uninfected Mv1Lu cells using PCR as detection method. A titre of 1×10^2 $TCID_{50}$/ml was measured. In parallel, human 293 kidney cells were inoculated with mink cell-derived PERV. Infection was shown by the presence of proviral DNA (Fig. 1), production of viral proteins and retroviral type C RT activity (Fig. 2, Table 1). In contrast to re-infected Mv1Lu cells, human 293 cells infected with the mink-adapted virus showed a higher RT activity after identical time periods post infection (Table 1).

PERV-induced morphological changes in cultured cells

Electron microscopy of PERV-infected mink cells revealed typical type C retroviruses with no obvious differences in comparison with PERV produced by infected human 293 cells (Fig. 3). However, in contrast to PERV-infected human 293 cells the cytoplasm of infected mink cells contained numerous multivesicular body (MVB)-like structures. These MVB contained numerous particles of virus-like morphology with a diameter of 60 nm (Fig. 4). Particles were smaller than intact type C virus particles and resembled in size and form the cores of immature retroviruses. Such MVBs were not detected in uninfected Mv1Lu cells. To examine whether MVBs contain viral proteins, immunofluorescence was performed using two antibodies, one against p15E (Fig. 5) and the other against p27Gag (data not shown) of PERV. Both antibodies revealed bright staining of vesicles in the cytoplasm, indicating that the MVBs contain PERV proteins. Uninfected cells remained negative in both assays. These results indicate that the MVBs contain at least two viral proteins, one capsid protein and one envelope protein, whereas in the nucleus and the surrounding cytoplasm viral proteins could not be detected.

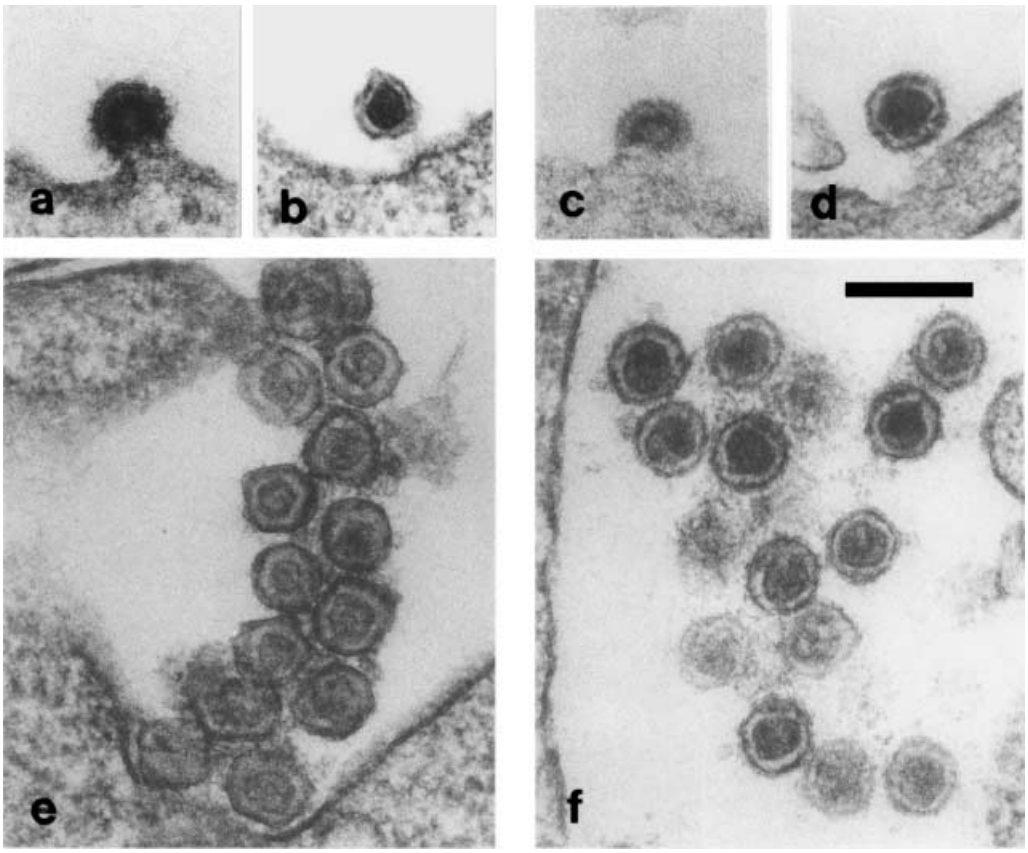

Fig. 3. Electron microscopy of budding or free PERV particles produced by human 293 cells (**a, b, e**) and mink Mv1Lu cells (**c, d, f**). Bar: 200 nm

Lack of PERV infection in American minks

To examine whether mink-adapted PERV is able to infect in vivo, three adult and three newborn American minks were inoculated with freshly pelleted virus preparations. In order to detect infection, sera from all animals were screened by Western blot for antibodies against PERV four weeks post infection and then every subsequent month. All animals were serologically negative at each time-point (Fig. 6). To detect whether a cryptic infection had taken place, DNA was isolated from blood cells of all adult animals 12 weeks post infection and tested for proviral DNA by nested PCR using PERV-specific primers. Amplification of PERV-sequences was seen in none of the samples (Fig. 7A). In addition, DNA was isolated from the ovaries, heart, kidneys, liver, spleen, brain, lungs, lymph nodes and pancreas of one mink euthanised 12 weeks after inoculation and screened for proviral DNA by nested PCR (Fig. 7B). In parallel, organs were screened for expression of viral proteins by immunohistology (data not shown). In all organs tested neither integration of proviral DNA nor expression of viral proteins was detected, indicating that no PERV infection had occurred.

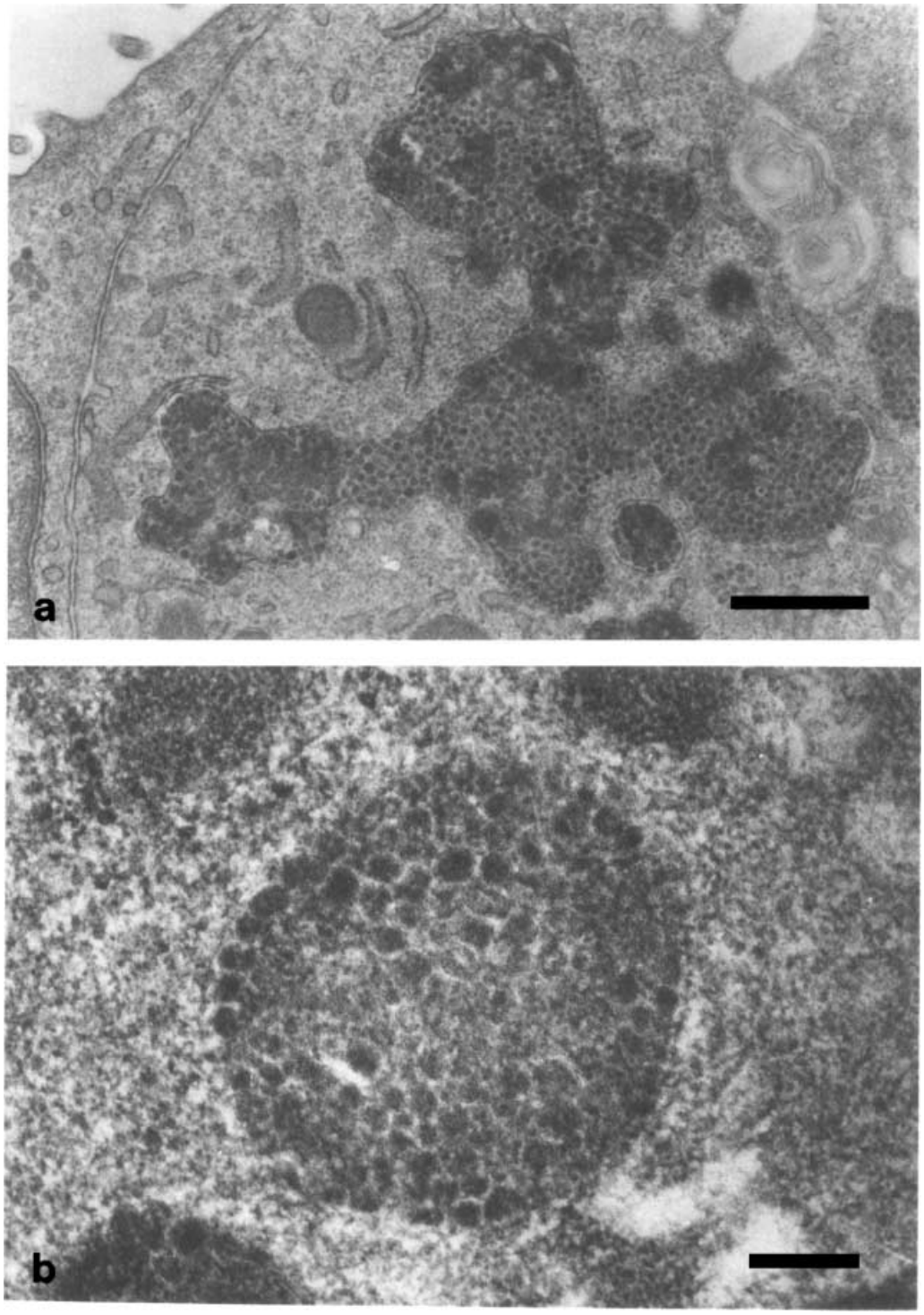

Fig. 4. Electron microscopy of PERV infected Mv1Lu cells showing multivesicular body (MVBs)-like structures. **a** Large cistern-like assembly of MVBs were seen all over the cytoplasma. Bar = 500 nm. **b** MVB containing numerous virus like particles (VLPs) with a diameter of 60 nm. Bar: 200 nm

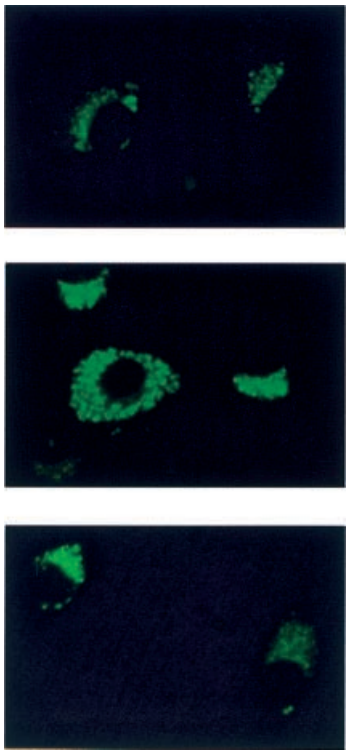

Fig. 5. Immunofluorescence microscopy of PERV infected Mv1Lu cells using a serum directed against p15E of PERV and a FITC-labeled secondary antibody. Staining visualized round cytoplasmatic structures representing MVBs. Using pre-immune serum or only the secondary antibody did not result in a staining of infected cells. Uninfected cells remained negative in each control. Microphotographs were take using a Zeiss Axiophot fluorescence microscope at a magnification of 300×

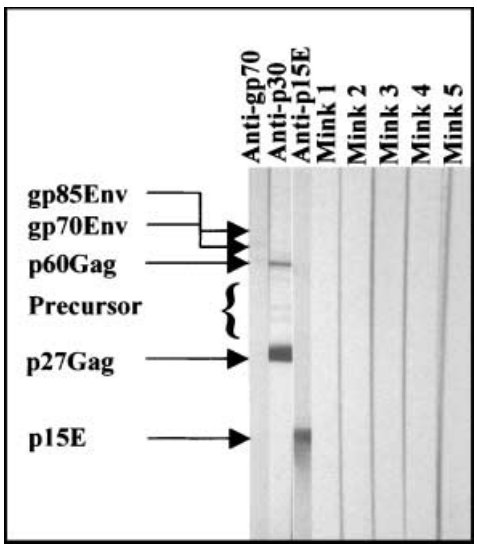

Fig. 6. Western blot using sera from 3 PERV-inoculated minks (1–3) and 2 control minks (4, 5) obtained 12 weeks post infection with PERV. To control the quality of the blot and the blotting procedure, sera against several structural proteins of PERV were included. None of the inoculated animals showed antibody reaction against PERV 12 weeks after virus application

Discussion

Numerous mammalian primary cells and cell lines have been shown to support productive infection by PERV [13, 14, 16, 20, 25, 32–34]. High virus titres were obtained with some cat and mink cells, among them the mink cell line

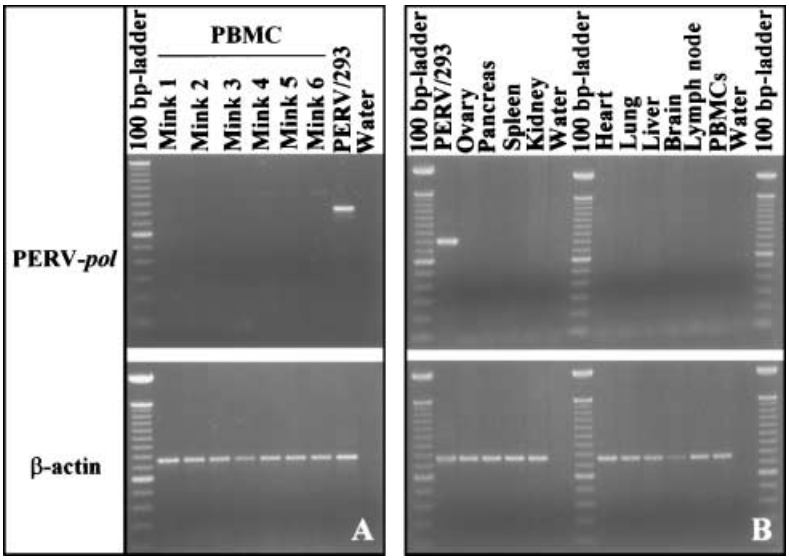

Fig. 7. Nested PCR with DNA isolated from **A** heparinized blood of minks and from **B** different organs of one mink (mink No. 3) 12 weeks post inoculation with PERV in vivo. PCR was performed using primers for PERV *pol*. To guarantee the quality of the DNA, primers for β-actin were used. In none of the tested organs provirus integration was observed. *M* 100 bp-ladder

Mv1Lu [26, 34]. Infection studies using pseudotypes showed the presence of the receptors for PERV-A and PERV-B on this cell line [32]. Using cell-free virus preparations of replication competent PERV/293, transmission of PERV-A and PERV-B was demonstrated by the presence of integrated provirus in the genome of Mv1Lu cells. When PERV derived from mink cells was incubated with human 293 cells, again both PERV-A and PERV-B were transmitted (Fig. 1), indicating that indeed both receptors are present on mink cells. It is important to note that among all human and animal cell lines tested until now, the human 293 kidney cell line best supports PERV replication. This property was not lost when 293 cells were infected with PERV derived from mink cells (Fig. 2, Table 1). Possibly, 293 cells possess transcription factors supporting PERV replication which are absent in other cells.

The multivesicular body (MVB)-like structures detected in the cytoplasma of PERV-infected Mv1Lu cells were clearly virus induced as they were absent in uninfected mink Mv1Lu cells and because they contain the viral proteins p27Gag and p15E. The nature of the MVBs and the virus-like particles (VLPs) inside the MVBs remains unclear. Morphologically similar MVBs containing small VLP had been described as normal components in neuronal and secretory cells. In addition, 30–50 nm VLPs were found in fetal bovine sera [4], in human breast tumours [10], in human milk [11, 24], in cell cultures obtained from patients with infectious mononucleosis [17] and in mice with a leucosis-like syndrome [6]. Neither the viral origin nor the physiological function of these MVBs and VLPs have been yet identified. The presence of viral proteins in the MVBs described here

(Fig. 5) and a limited morphological similarity to viral structures (Fig. 4) suggest that these particles may represent defective viruses. In addition, the low titre of PERV released from infected Mv1Lu cells indicate a low frequency of mature infectious virus particles and support the idea that immature PERV particles may accumulate in these multivesicular bodies. The morphological changes in the infected cells indicate that pathological changes may occur also in vivo.

An endogenous mink retrovirus (MiLV) was found to be released from Mv1Lu cells after long-term passage or after co-culture with BrdU-treated mouse cells [23]. The absence of virus particles in electron micrographs (not shown) and the absence of RT activity in uninfected mink cells indicate that the Mv1Lu cell line used for these infection studies does not release endogenous mink virus. However, recombinations at the level of proviral DNA or genomic RNA may have occurred, resulting in the production of MVBs containing PERV proteins and VLPs.

Because PERV seems to infect a wide range of mammalian cells in vitro [26, 32, 34], it is important to know whether PERVs can infect the corresponding species in vivo. Since initial attempts to infect rats and guinea pigs did not result in infection of these animals [26], a virus-host-model was chosen, in which the virus used for infection was already adapted to the given species. Retroviruses are known to incorporate numerous cellular proteins during the budding process, among them histocompatibility antigens. PERV grown on mink cells should have derived its lipid membrane from mink cells and should have included cellular mink proteins during budding. PERV adapted in such a way was expected to better overcome the immune responses of the inoculated host and to better replicate in minks. However, even mink-cell adapted PERV failed to productively infect adult or newborn minks.

The recent demonstration that severe combined immunodeficient (SCID) mice can be infected with PERV released from implanted porcine pancreatic islets [5, 12], shows that trans-species transmission of PERV in vivo is possible. It therefore cannot be excluded that inoculation of larger amounts of virus or infection during pharmacological immunosuppression (simulating the situation in xenotransplantation) could lead to infection of minks in vivo. Apart from the differences in species, possible reasons for the difference in outcome include the virus subtype and titre used, the use of cells instead of cell-free virus and the complete lack of a functional immune system in the SCID mouse. Investigations in rhesus monkeys inoculated intravenously with cell-free SIV showed the viral half-life to be only a few minutes [35], indicating the importance of the innate immunity. Since human immunodeficiency virus (HIV) can infect very effectively via cell-cell contact [3], the transmission of PERV from porcine pancreatic islets to SCID mice might similarly result from virus budding directly from the porcine cell into the murine tissues. When using transplants from transgenic pigs engineered to express human complement regulatory proteins to avoid hyperacute rejection, PERV particles resistant to the human complement system might be released by the transplant. In the absence of normal immune responses, these viruses could replicate and induced diseases characteristic for retrovirus

infections such as tumours and immunodeficiencies [7, 18, 22]. Further and more detailed investigations in small animals and non-human primates must therefore be performed to evaluate the potential risk of PERVs during xenotransplantation.

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