Neutralization of porcine endogenous retrovirus by antibodies against the membrane-proximal external region of the transmembrane envelope protein

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Immunization of different species including goats, rats, hamsters and guinea pigs with the recombinant ectodomain of the transmembrane envelope (TM) protein p15E of porcine endogenous retrovirus (PERV) has been shown to result in the production of virus-neutralizing antibodies. The sera recognize two groups of epitopes, one located in the fusion peptide-proximal region (FPPR) and the second in the membrane-proximal external region (MPER) of p15E. Most interestingly, the epitopes in the MPER are similar to epitopes in the TM protein gp41 of human immunodeficiency virus type 1 (HIV-1) recognized by mAbs 2F5 and 4E10, which broadly neutralize HIV-1. To study which epitope and which antibody population are involved in the process of neutralization of PERV, this study generated a new antiserum in a goat using an elongated ectodomain of p15E. The immune serum neutralized PERV at a higher titre and recognized broader epitopes in the FPPR and MPER of p15E. For the first time, antibody subpopulations were isolated from this serum using affinity chromatography with immobilized proteins and peptides corresponding to the FPPR and MPER of p15E. Only the affinity-purified antibodies specifically binding the MPER neutralized PERV, indicating that, as in the case of HIV-1, the MPER is an important target of neutralizing activity.

INTRODUCTION

The induction of neutralizing antibodies is a promising way to protect against retroviral infections. With the spread of the AIDS pandemic, there is an increasing need to develop vaccines protecting against human immunodeficiency virus type 1 (HIV-1) infection. Numerous antibodies broadly neutralizing HIV-1 have been described (Walker & Burton, 2010), among these mAbs 2F5 and 4E10, both directed against the membrane-proximal external region (MPER) of the transmembrane envelope (TM) protein gp41 of HIV-1 (Muster et al., 1993; Zwick et al., 2001). However, to date, all attempts to induce such broadly neutralizing antibodies in immunization studies have failed (Denner, 2011; Montero et al., 2008; Munier et al., 2011).

In contrast with these results, immunization of goats, cats and guinea pigs with the recombinant ectodomain of the TM protein p15E of different gammaretroviruses such as porcine endogenous retrovirus (PERV) (Fiebig et al., 2003; Kaulitz et al., 2011), feline leukemia virus (FeLV) (Langhammer et al., 2005, 2006, 2011a, b) and koala retrovirus (KoRV) (Fiebig et al., 2006) effectively induces neutralizing antibodies. The induced antibodies recognize epitopes located in the fusion peptide-proximal region (FPPR) and MPER of p15E. Despite the evolutionary differences between HIV-1 and gammaretroviruses, sequence homology was observed in the MPER of p15E of PERV, FeLV and KoRV (FEGWFN) and the mAb 4E10-targeted epitope in the MPER of gp41 of HIV-1 (NWFN/DIT, identical amino acids underlined). In the case of p15E-induced neutralizing antibodies, it is still unclear which epitopes and antibodies are involved in neutralization and how these antibodies neutralize the virus.

During infection, the TM proteins of retroviruses undergo conformational changes bringing the FPPR and MPER into close proximity (Gallo et al., 2003; Pan et al., 2010). In addition, in the case of HIV-1, an interaction between the MPER and FPPR of gp41 has been shown (Bellamy-McIntyre et al., 2007; de la Arada et al., 2009; Lay et al., 2011). Furthermore, the binding of 2F5 and 4E10 to a peptide containing their epitopes was increased in the presence of a peptide corresponding to the FPPR (Fiebig et al., 2009). An understanding of the interaction of the MPER and FPPR during infection, neutralization and induction of neutralizing antibodies may be helpful in designing antigens able to induce neutralizing antibodies.

Here, antibodies against an extended version of the recombinant ectodomain of p15E of PERV were induced. The antibodies were analysed for neutralization and epitope binding. In order to study the contribution of different subpopulations to neutralization, the antibody populations were separated by affinity chromatography using recombinant proteins and synthetic peptides corresponding to the
epitopes in the FPPR and MPER. Whereas antibodies specific for the MPER had neutralizing activity, those against the FPPR did not neutralize PERV.

**RESULTS**

**Generation of p15E-specific antibodies and determination of their neutralizing activity**

To generate p15E-specific antibodies, a new construct corresponding to the ectodomain (aa 478–603) of p15E of PERV [p15E(478–603), Fig. 1], was cloned, expressed and purified by affinity chromatography (Fig. 2a). In contrast to the previously used p15E ectodomain comprising aa 488–596 [p15E(488–596), Fig. 1], p15E(478–603) was extended for a better presentation to the immune system of the FPPR as well as of the MPER epitope FEGWFN (Fig. 1). A neutralizing antiserum specific for the shorter p15E(488–596) (Kaulitz et al., 2011) was shown to react with the purified protein (Fig. 2a). The glutathione S-transferase (GST) tag was removed and gel filtration analysis showed that the tagless antigen p15E(488–603) used for immunization produced dimers under physiological conditions (Fig. 2b). In contrast, the previously used fusion protein of calmodulin binding protein (CBP) and aa 488–596 of p15E [CBP–p15E(488–596)] formed only monomers under the same conditions (Fig. 2b). The immune serum from goat 350 reacted with the antigen used for immunization as well as with recombinant proteins corresponding to the NHR and CHR of p15E in a Western blot analysis (Fig. 2a), indicating that binding antibodies specific for both parts of the protein were induced. The immune serum and p15E-specific antibodies purified by affinity chromatography using immobilized recombinant p15E showed binding to FPPR- and MPER-derived peptides with titres in the range of $5 \times 10^5$ for the serum and $10 \mu$g ml$^{-1}$ for p15E-specific antibodies (Fig. 2c, d).

In a neutralization assay measuring the reduction in infection by real-time PCR, the serum was found to neutralize at a dilution as low as 1:100. The p15E-specific antibodies isolated by affinity chromatography neutralized at concentrations as low as 80 $\mu$g ml$^{-1}$ (Fig. 2e), indicating that these antibodies were responsible for the neutralization.

Finally, epitope mapping showed the main epitopes in the FPPR and MPER (Fig. 3). In contrast to previous immunizations with shorter recombinant ectodomains, the sequence recognized in both regions was larger. As one epitope usually contains six to eight amino acids, the number of epitopes was obviously higher. The main epitope in the FPPR was defined by the measurement of $80 \times 10^5$ fluorescence units. In addition, a minor epitope was found in the C–C loop region of p15E.

**Comparison of three goat sera induced by immunization with p15E**

To compare the antiserum against the extended p15E(478–603) with antisera produced after immunization with the shorter p15E(488–596), ELISA and neutralization assays were performed. Epitope mapping of goat 350 serum showed similarly located epitopes, although they were extended (Fig. 4a). ELISA showed that goat 350 serum had the highest titre of FPPR- and MPER-specific antibodies (Fig. 4b). This result was confirmed by surface plasmon resonance binding analysis (data not shown). Finally, isolated IgG from goat 350 serum showed the highest neutralization activity in comparison with IgGs isolated from goat 346 and goat 355 sera (Fig. 4c).

**Isolation of FPPR- and MPER-specific antibodies**

In order to analyse which of the antibodies directed against p15E were responsible for neutralization, FPPR/NHR- and MPER/CHR-specific antibodies were isolated using affinity
Neutralization of PERV

The serum was analysed, the epitopes in the MPER and FPPR were defined and, for the first time, the immune serum used as a control was non-neutralizing. Two flowthrough fractions and two eluates obtained by affinity chromatography using immobilized recombinant proteins (GST–FPPR/NHR and GST–MPER/CHR4K) were analysed in the neutralization assay. When the GST–FPPR/NHR column was used, the flowthrough fraction contained only MPER-specific antibodies and was neutralizing. In contrast, when the GST–MPER/CHR4K column was used, the flowthrough fraction did not contain MPER-specific antibodies and was not neutralizing (Fig. 5), whereas the flowthrough contained FPPR/NHR-specific antibodies (Fig. 5, Table 1).

Neutralizing activity of FPPR- and MPER-specific antibodies

Immune serum of goat 350 and the p15E-specific antibodies purified by affinity chromatography were shown to inhibit infection of human embryonic kidney (HEK) 293 cells with PERV (Fig. 2e). The isolated IgG fraction from the pre-immune serum used as a control was non-neutralizing. Two flowthrough fractions and two eluates obtained by affinity chromatography using immobilized recombinant proteins (GST–FPPR/NHR and GST–MPER/CHR4K) were analysed in the neutralization assay. When the GST–FPPR/NHR column was used, the flowthrough fraction contained only MPER-specific antibodies and was neutralizing. In contrast, when the GST–MPER/CHR4K column was used, the flowthrough fraction did not contain MPER-specific antibodies and was not neutralizing (Fig. 6a). When the eluates were analysed, the eluate from the column with immobilized GST–MPER/CHR4K contained MPER-specific antibodies and was neutralizing; in contrast, the eluate from the GST–FPPR/NHR column did not neutralize the virus (Fig. 6b; for titres see Table 1).

When affinity chromatography was performed using the synthetic MPER-derived peptide p15E(585–606) immobilized on AminoLink columns, the eluate contained only MPER-specific antibodies (Fig. 5b) and was neutralizing at concentrations as low as 7.5 μg ml⁻¹ (Fig. 6c).

**DISCUSSION**

Antibodies neutralizing PERV were induced after immunization with an extended ectodomain of its p15E protein. The serum was analysed, the epitopes in the MPER and FPPR were defined and, for the first time, the immune serum was fractionated using affinity chromatography with the immobilized recombinant proteins GST–MPER/CHR4K [p15E(562–603)] and GST–FPPR/NHR [p15E(478–532)], and MPER-specific antibodies were isolated using a synthetic MPER-derived peptide [p15E(585–606)] (Fig. 1). A synthetic FPPR-derived peptide was not used for affinity chromatography. The flowthrough and the eluted fractions were collected and characterized for binding and neutralizing antibodies (Figs 5 and 6).

The ELISA showed that eluates or flowthrough fractions from all experiments contained predominantly only one population of antibodies, directed against either the MPER or the FPPR. Flowthrough fractions from the column with immobilized GST–FPPR/NHR contained CHR/MPER-specific antibodies, whereas the eluate contained FPPR/NHR-specific antibodies. Eluates from columns with immobilized GST–MPER/CHR4K contained CHR/MPER-specific antibodies and from columns with the immobilized MPER-derived peptide contained only MPER-specific antibodies (Fig. 5), whereas the flowthrough contained FPPR/NHR-specific antibodies (Fig. 5, Table 1).
recombinant proteins corresponding to the C- and N-terminal parts of p15E, as well as with a synthetic peptide corresponding to the MPER. The results showed that only antibodies directed against the MPER were neutralizing.

The extended ectodomain was designed to present the FPPR/MPER epitopes in a better way to the immune system. In comparison with previous immunizations with smaller variants of the ectodomain, higher titres of antibodies directed to the FPPR- and MPER-derived peptides were achieved, which also resulted in a better neutralization activity (Fig. 4b, c). The differences in the titres and affinity of the sera may be explained by the absence of a tag protein, by the ability to form dimers under physiological conditions (Fig. 2b) and/or differences in the length of the recombinant protein (Fig. 4a). The sequences recognized by the immune serum from goat 350 in the FPPR and MPER were extended in comparison with the epitopes recognized by sera induced by the smaller proteins and were certainly representing different overlapping epitopes. The main epitope in the MPER recognized by goat 350 serum was defined as EGWFNR (amino acids identical to HIV-1 underlined) and was identical to the epitope recognized by goat 355 serum as well as the epitopes recognized by sera from immunized rats, mice and guinea pigs (Fiebig et al., 2003; Kaulitz et al., 2011).

This epitope is localized similarly to the epitope NWFNDIT, which is recognized by mAb 4E10 isolated from HIV-1-infected individuals and neutralizes up to 95% of all HIV-1 strains (Binley et al., 2004). Using the 2F5 and 4E10 mAbs that broadly neutralize HIV-1 and antibodies neutralizing PERV in HIV-1-specific and PERV-specific neutralization assays, as well as in Western blot assays, no cross-neutralization or cross-reactivity was observed (data not shown). With regard to the antibodies binding the minor epitope containing the C-C loop region of p15E (Fig. 3), we could not exclude the possibility that they are also neutralizing, as was described for mAb 7C4 binding to this region (Chiang et al., 2007).

Similar epitopes in the FPPR and MPER have been detected after immunization of different species including goats, rats and cats with the ectodomain of the TM protein p15E of FeLV (Langhammer et al., 2005, 2006, 2011a, b).

In this study, we approached a better understanding of the mechanism of neutralization by antibodies specific for p15E of PERV by answering the question of which antibodies were involved in neutralization. When FPPR- and MPER-specific antibodies were isolated and tested for binding properties, the ELISA results showed that it was possible to separate the two subpopulations (Fig. 5). When MPER- and FPPR-specific antibodies were analysed for neutralization, only MPER-specific antibodies were neutralizing. In this region, EGWFNR was mapped as the major epitope; therefore, it is likely that the corresponding antibodies are involved in neutralization. This assumption is supported by previous data showing that an MPER-derived peptide reduced the inhibition of PERV infection by an antiserum induced by immunization with p15E(488–596) (Kaulitz et al., 2011). In contrast, a FPPR-derived peptide had no effect on the neutralizing activity of the antiserum.

As it appears to be easy to induce neutralizing antibodies following immunization with the ectodomain of p15E of different gammaretroviruses (Fiebig et al., 2006; Kaulitz et al., 2011; Langhammer et al., 2006), the question remains as to why is it so difficult to obtain neutralizing antibodies when immunizing with the ectodomain of the TM proteins of lentiviruses such as HIV-1 (Law et al., 2007; Mantis et al., 2001; Nieva et al., 2011) and HIV-2 (Behrendt et al., 2012). Glycosylation, the interaction...
between the MPER and the lipid bilayer, and/or the conformation of the molecule may be important (Ma et al., 2011; Montero et al., 2008; Van Regenmortel, 2011). First attempts to immunize with the MPER of HIV-1 using scaffold proteins or mutated six-helix bundles have indicated that such strategies may induce neutralizing antibodies, and this suggests that the conformation may be important (Arnold et al., 2009; Wang et al., 2011).

The neutralization assay used here was based on the measurement of viral DNA by real-time PCR, thus clearly showing that the cells were infected and that the reverse transcriptase was active. A decreased amount of viral DNA indicates neutralization. This assay has numerous advantages: (i) it is sensitive as it is based on PCR, (ii) it is quick, measuring an early event in infection, and (iii) using a 96-well setting, it needs only a small amount of serum and can measure many samples or fractions at the same time.

The result that MPER-directed antibodies induced by different gammaretroviruses as well as HIV-1 have neutralizing activity suggests that this region is exposed during infection of all retroviruses. Therefore, an understanding of the mechanism of neutralization of PERV and other gammaretroviruses may help to design gp41-derived antigens able to induce neutralizing antibodies to HIV-1.

**METHODS**

**Cloning, expression and purification of recombinant proteins.**

Three sequences: (i) the ectodomain of the TM envelope protein p15E of PERV-A (NCBI protein accession no. CAA72927) lacking the FP (aa 478–603), (ii) the CHR of p15E (aa 562–603) and (iii) the NHR (aa 478–532) were amplified and cloned into the pGEX-KG vector (Fig. 1). The GST-tagged proteins were expressed in *Escherichia coli*.

**Fig. 4.** Characterization of the binding and neutralizing activity of sera from goats immunized with different recombinant p15E proteins. (a) Comparison of the epitopes recognized by goat 346, 355 and 350 sera. The antigens used for immunization are shown as grey shaded bars. Goats 346 and 355 were immunized with p15E(488–596) and goat 350 with p15E(478–603). The main epitopes were recognized in the FPPR and MPER (filled bars). The additional peptides recognized by goat serum 350 in the N terminus are indicated by an open bar. (b) Titration of purified Igs from goat 346 (●), 355 (●) and 350 (▲) sera for binding to peptides corresponding to the FPPR (dashed line) and MPER (solid line). (c) Neutralizing activity of the goat sera (filled bars, goat 355; light grey bars, goat 346; dark grey bars, goat 350) measured as inhibition of provirus integration. The reduction in provirus load was calculated using the ΔCt of PERV PERV-A DNA and GAPDH DNA. The pre-immune serum of goat 350 was used as a negative control (open bars).

**Fig. 5.** Titration of antibodies binding to FPPR-derived peptide p15E(478–500) (▲) and MPER-derived peptide p15E(585–606) (●) in an ELISA. In the assays, purified antibodies as well as flowthrough fractions obtained by affinity chromatography of goat 350 serum using immobilized recombinant proteins corresponding to the CHR and NHR of p15E (a) and the immobilized MPER-derived peptide (b) were analysed.
GST–MPER/CHR4K, respectively) were used for affinity-chromatography columns. Four lysines (K) were added to the GST–MPER/CHR protein to increase the solubility of the recombinant fusion protein. The purity of the proteins was analysed by SDS-PAGE; the protein bands in the SDS gel were stained with Coomassie Brilliant Blue and Western blot analysis was performed using serum from another goat immunized with p15E (Kaulitz et al., 2011). The protein concentration was measured using dilutions of BSA in SDS-PAGE analyses and by absorption at 280 nm using the calculated molar adsorption coefficients $e_1=1.284$, $e_2=1.052$ and $e_3=1.653$ mol$^{-1}$ cm$^{-1}$ for p15E(aa 478–603), GST–FPPR/NHR and GST–MPER/CHR4K, respectively. The CBP–p15E(488–596) fusion protein was produced and purified as described previously (Kaulitz et al., 2011).

**Synthetic peptides.** Peptides (Fig. 1) were synthesized by Genaxxon Bioscience. The peptides were dissolved in water or PBS.

**Immunization and isolation of Igs.** Goat 350 was immunized twice (at weeks 0 and 3) with 500 µg purified recombinant protein p15E(478–603) emulsified in Freund’s adjuvant (first immunization with complete adjuvant and second immunization with incomplete adjuvant) by two subcutaneous injections in the back. Serum was taken at weeks 3, 6 and 9 after first immunization and decomplemented by heating at 56°C for 30 min. To isolate Igs, equal amounts of goat serum and 4 M ice-cold ammonium sulfate in water were mixed and stirred for 30 min at 4°C. After centrifugation (10000 g, 10 min, 4°C), the pellet was resuspended in two-thirds of the initial volume of the goat serum in PBS and the antibodies were precipitated again with an equal volume of 4 M cold ammonium sulfate. After the second precipitation, the pellet was resuspended in one-fifth of the initial volume and dialysed against PBS.

**Western blotting and ELISA.** For Western blot analysis, the recombinant proteins p15E, GST–FPPR/NHR and GST–MPER/CHR4K were subjected to SDS-PAGE using 15% acrylamide and Tricine buffer (Schägger & von Jagow, 1987) and transferred to a PVDF membrane. After blocking in PBS with 0.1% Tween 20 with 5 % (w/v) non-fat dried milk, the membrane was incubated with antisera (diluted 1:1000) and subsequently incubated with HRP-conjugated anti-goat antibody (diluted 1:1000; Dako) and developed using an ECL detection system (Thermo Scientific).

For ELISA, 100 ng peptide per well was coated onto microtitre plates (Greiner Bio One). Serum or purified antibody was added to wells blocked with 5% BSA in PBS with 0.1% Tween 20. An anti-goat HRP-labelled antibody (diluted 1:2000; Dako) was added together with 80 µl o-phenylenediamine dihydrochloride (1 mg ml$^{-1}$, Sigma) for visualization. Colour development was stopped with sulfuric acid and the result was measured photometrically at 492 nm using a 96-well plate reader (TECAN Spectra classic) using absorbance at 620 nm as a reference.

**Gel filtration.** The purified TM proteins p15E(478–603) and CBP–p15E(488–596) (Kaulitz et al., 2011) were subjected to gel filtration in a Superdex 200 10/300 GL column using Äktta Explorer 10S (GE Healthcare). PBS was used as the running buffer.

**Epitope mapping.** For the identification of binding sites of the induced antibodies, a PepStar microarray chip (IPT Peptide Technologies) based on glass slides spotted with 15mer peptides overlapping by 12 aa in triplicate was used. Mapping was performed according to the manufacturer’s instructions. Briefly, the slides were incubated with goat serum (diluted 1:500) at 4°C overnight. After washing with TBS, the slides were incubated with secondary antibody (DyLight 649-conjugated AffiniPure rabbit anti-goat, diluted 1:500; Jackson ImmunoResearch) and washed five times with TBS and five times with deionized water for 5 min each. For drying, the slide was centrifuged at 500 g for 1 min. The fluorescence scan was performed using a GenePix 4000B Scanner (Molecular Devices).

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**Fig. 6.** (a, b) Neutralizing activity of antibodies purified by affinity chromatography using GST–FPPR/NHR and GST–MPER/CHR4K coupled to cyanogen bromide (CNBr) activated Sepharose in flowthrough fractions (a) and eluted fractions (b). Fractions containing FPPR-specific antibodies are shown as open bars and fractions containing MPER-specific antibodies as dark grey bar. Pre-immune serum and IgG isolated from the pre-immune serum fractions containing MPER-specific antibodies as filled bars. Fractions containing MPER-specific antibodies as light grey bars were used as a negative control, and whole goat serum 350 µl and p15E-specific antibodies (filled bars) as a positive control. (c) Neutralizing activity of antibodies purified by affinity chromatography using the MPER-derived peptide p15E(562–606).

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BL-21 by induction at 37°C in Luria–Bertani medium with 0.1 mM IPTG. The bacteria were lysed using lysozyme and sonication. The soluble proteins were purified by affinity chromatography using glutathione-Sepharose 4B (GE Healthcare) and p15E was eluted by cutting with thrombin (GE Healthcare) to remove the GST tag. The recombinant proteins FPPR/NHR [p15E(aa 478–532)] and MPER/CHR4K [p15E(aa 562–603)] tagged with GST (GST–FPPR/NHR and GST–MPER/CHR4K, respectively) were used for affinity-chromatography columns. Four lysines (K) were added to the GST–MPER/CHR protein to increase the solubility of the recombinant fusion protein. The purity of the proteins was analysed by SDS-PAGE; the protein bands in the SDS gel were stained with Coomassie Brilliant Blue and Western blot analysis was performed using serum from another goat immunized with p15E (Kaulitz et al., 2011). The protein concentration was measured using dilutions of BSA in SDS-PAGE analyses and by absorption at 280 nm using the calculated molar adsorption coefficients $e_1=1.284$, $e_2=1.052$ and $e_3=1.653$ mol$^{-1}$ cm$^{-1}$ for p15E(aa 478–603), GST–FPPR/NHR and GST–MPER/CHR4K, respectively. The CBP–p15E(488–596) fusion protein was produced and purified as described previously (Kaulitz et al., 2011).

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Affinity chromatography for isolation of antibody subpopulations. To isolate NHR/FPPR- and CHR/MPER-specific antibodies, the recombinant proteins GST–FPPR/NHR and GST–MPER/CHR4K were conjugated to CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. The serum was incubated with the conjugated matrix at 4 °C overnight. Unbound serum was washed away with PBS. The bound antibodies were eluted with 0.2 M glycine/HCl (pH 2.1) and dialysed against PBS. In addition, p15E-specific antibodies were isolated using recombinant GST–p15E(478–603) conjugated to CNBr-activated Sepharose. To analyse the CHR-specific antibodies in more detail, 2 mg MPER-derived peptide (aa 585–606) was coupled to 1 ml AminoLink resin (Thermo Scientific) according to the manufacturer’s instructions. Binding and elution of specific antibodies were performed as described for purification using CNBr-activated Sepharose.

Neutralization assay and real-time PCR. Neutralization assays were performed using cell-free supernatants produced by HEK293 cells infected with a recombinant PERV-A/C repeatedly passaged on human cells. This virus is associated with higher titres and genetic alterations in its long-terminal repeats (Karlas et al., 2010).

Uninfected HEK293 cells (100 μl containing 30 000 cells ml⁻¹) were seeded into each well of a 96-well microtitre plate (Greiner) and incubated for 24 h at 37 °C in 5 % CO₂. Different serum dilutions (in 50 μl medium) were mixed with 50 μl infectious PERV, incubated for 30 min at 37 °C and added to the cells. After incubation for 72 h at 37 °C, the cells were examined by light microscopy for viability and the medium removed. The cells were lysed by freezing and thawing three times for 10 min each at −80 °C and 95 °C, respectively, followed by incubation with lysis buffer [nuclease-free water containing 0.2 mg proteinase K ml⁻¹ and 10 % (v/v) 10⁻⁶ PCR buffer] at 60 °C for at least 4 h. The proteinase K was heat inactivated for 30 min at 95 °C, and 3 μl lysate was used in a duplex real-time PCR to measure proviral DNA transcribed from the genomic viral RNA by the reverse transcriptase. The primer sequences are given in Table 2.

Neutralization was measured as the reduction in viral DNA in cells in the presence of immune serum/isolated antibodies in comparison with the pre-immune serum, as determined by duplex real-time PCR using a specific PERV gag gene probe and a human gapdh gene probe as a control gene (Kaulitz et al., 2011) (Table 1). The primer and

Table 1. Overview of the binding and neutralizing activity of the purified serum fractions

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<th>Fraction</th>
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*Results are shown as dilution for the flowthrough and μg ml⁻¹ for the eluate.

Table 2. Primer and probes used in this study

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<td>CHR4K rev</td>
<td>TTCTCAGCATTCTTCTTCTGCTCATCCAAGG</td>
<td>−7944 to −7958</td>
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</tbody>
</table>

*HEX, Hexachlorofluorescein; BHQ1, Black Hole Quencher 1; FAM, fluorescein.
†Forward primers contained an EcoRI site and reverse primers contained a XhoI site (underlined). Two Ts were added at the start of the sequence to maintain the reading frame. In the reverse primers, a CTA stop codon was added after the restriction site. The sequence TTTCCTTCTCTTATTGCTCATCAAAGG encodes four lysines.
probes sequences are given in Table 2. The difference in provirus load was calculated using the cycle threshold ($C_t$) values of PERV-A (gene of interest) and GAPDH (control gene): $\Delta C_t = C_t(\text{PERV-A}) - C_t(\text{GAPDH})$. The $C_t$ value was defined as the number of cycles required for the fluorescent signal to cross the threshold. A PERV $C_t$ value of $25-26$ was considered to represent optimal infection, at the same time that GAPDH showed a $C_t$ value of $24-25$. The cut-off was determined by adding $1$ $C_t$ to the $C_t$ value of the pre-immune serum (i.e. a $\Delta C_t$ value of $1$ represented a 50% reduction).

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**REFERENCES**


