Mapping of a neutralizing epitope in the surface envelope protein of porcine endogenous retrovirus subgroup B

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Pigs are thought to be the most suitable donor animal for xenotransplantation. However, pigs harbour potentially hazardous infectious agents, termed porcine endogenous retroviruses (PERVs), in their genome. In this study, we generated a mAb against PERV-B surface (SU) envelope protein (Env), designated KRT1. KRT1 binding was detected by an indirect immunofluorescence assay and flow cytometric analysis on cells infected with PERV-B. KRT1 neutralized PERV-B pseudotype virus and specifically recognized PERV-B SU Env, but not PERV-A SU Env by immunoblotting analysis. The peptide-ELISA revealed that KRT1 recognized a linear peptide sequence (ALEPPHNLPVP) residing in a proline-rich region that is one of the subdomains of SU Env. In conclusion, the KRT1 antibody will serve as a useful tool for the study of PERV-B and, more importantly, it may provide new protective strategies against PERV-B infection in xenotransplantation.

Xenotransplantation is a solution to the shortage of human donors, and pigs are considered as the most suitable donor animal (Blusch et al., 2002). Although specific-pathogen-free pigs will be used for xenotransplantation, all pigs possess potentially hazardous infectious viral agents, named porcine endogenous retroviruses (PERVs), in their genome (Blusch et al., 2002). PERVs are members of the genus Gammaretrovirus and are divided into three subgroups (termed PERV-A, PERV-B and PERV-C) based on their different receptor usages (Le Tissier et al., 1997; Patience et al., 1997; Denner, 2008). PERV-A and PERV-B may transmit from pigs to humans, because humans have functional receptors for both PERV-A and PERV-B (Takeuchi et al., 1998; Denner, 2008). Although the risks caused by the infection with PERVs have not been fully assessed at present, they may potentially cause leukaemia, anaemia, immunodeficiency and cancers like other gammaretroviruses (Kawakami et al., 1980; Wilson et al., 2000; Denner, 2008).

Gammaretroviral envelope (Env) consists of surface (SU) and transmembrane (TM) subunits, which interact via a disulfide bond between SU Cx2C- and TM Cx2C-motifs (Wallin et al., 2004). PERV SU Env consists of four subdomains, known as variable regions A and B (VRA and VRB), proline-rich region (PRR) and the C-terminal region (CR). Of these domains, VRA, VRB and PRR are divergent between subgroups (Le Tissier et al., 1997; Watanabe et al., 2005). In ecotropic and amphotropic murine leukaemia viruses, the receptor-binding domain needed for binding with host receptors spans VRA and VRB. In PERV-A and PERV-B, PRR might be involved in the proper binding to their receptors together with VRA and VRB (Watanabe et al., 2005). Recently, it was revealed that CR was also important for the infectivities of PERV-A and PERV-C (Gemeniano et al., 2006; Argaw et al., 2008).

The information about the antigenicity of PERV Env is quite limited (Chiang et al., 2007) and the antigenic property of PERV-B Env has not yet been reported. In this study, we generated a neutralizing mAb against PERV-B and determined the neutralizing epitope.

Human embryonic kidney (HEK) 293 (CRL-1573; ATCC) cells chronically infected with PERV-B (HEK293/PERV-B) (Le Tissier et al., 1997), HEK293T (CRL-11268; ATCC), TELCeB6 (Cosset et al., 1995), TELCeB6/FBPERV-A (Takeuchi et al., 1998), TELCeB6/FBPERV-B (Takeuchi et al., 1998), TELCeB6/FBFeLV-B (FeLV-B, feline leukaemia virus subgroup B) (Nakata et al., 2003) cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated FCS, 100 IU penicillin (Invitrogen) ml⁻¹ and 100 μg streptomycin (Invitrogen) ml⁻¹ at 37 °C in a humidified atmosphere of 5% CO₂ in air. P3U1 (mouse myeloma) cells were cultured in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine (Sigma), 100 IU penicillin (Invitrogen) ml⁻¹ and 100 μg streptomycin (Invitrogen) ml⁻¹ at 37 °C in a humidified atmosphere of 5% CO₂. Insect cell lines, Sf9...
(Invitrogen) and HighFive cells (Invitrogen) were cultured at 27°C without exchanging the air in SF900II and ExpressFive (Invitrogen), respectively, which were supplemented with 18 mM l-glutamine (Sigma) and 10 μg gentamicin (Invitrogen) ml⁻¹.

For immunoblot analysis, samples were subjected to SDS-PAGE using a commercial gradient gel, and the separated proteins were blotted onto 0.2 μm PVDF (Bio-Rad Laboratories).

PERV-A and PERV-B SU Env tagged with 6× histidine (His) at the C terminus (termed PASUFull-HIS and PBSUFull-HIS, respectively) were expressed using a baculovirus expression system as described previously (Watanabe et al., 2005). PBSUFull-HIS was purified with a HisTrap HP column (GE Healthcare). Purified protein was subjected to Coomassie brilliant blue (CBB) staining and immunoblot analysis using an anti-His mAb (R&D Systems) (Fig. 1a). By both CBB staining and immunoblot analysis, we detected a 65 kDa protein corresponding to the expected size of the PBSUFull-HIS protein. Fifty micrograms of the purified protein with Freund’s complete adjuvant was immunized intracutaneously four times every 2 weeks in BALB/c mice. One week after the fourth immunization, 25 μg of the purified protein was immunized intravenously. Three days after the final immunization, splenocytes were then harvested and fused with the P3U1 cells to generate hybridoma cells. Hybridoma cells were cloned, and each culture supernatant was subjected to an ELISA to select clones which reacted with PBSUFull-HIS (data not shown). A selected clone, named KRT1, was inoculated into BALB/c mice to generate ascites. Harvested

Fig. 1. Generation of a neutralizing antibody against PERV-B. (a) Purification of a recombinant PERV-B Env fused with a His-tag (PBSUFull-HIS). The purified protein was detected by CBB staining and an immunoblot analysis using an anti-His mAb. (b) Detection of PERV-B antigen by IFA. HEK293 and HEK293/PERV-B cells were subjected to IFA using the mAb KRT1 and a normal mouse IgG (negative control, N.C.) as the first antibody. Cells were observed by phase-contrast microscopy (PCM) and fluorescent microscopy (FM). (c) Detection of PERV-B antigen by flow cytometric analysis. HEK293 and HEK293/PERV-B cells were subjected to flow cytometry using the mAb KRT1 and a normal mouse IgG (N.C.) as the first antibody. The filled histograms and the blank histograms indicate HEK293 and HEK293/PERV-B cells, respectively. Vertical and horizontal axes indicate numbers of cells and fluorescent intensities, respectively. (d) Neutralizing activity of the mAb KRT1. KRT1 and a normal mouse IgG (N.C.) were subjected to the neutralization assay using pseudotype viruses of PERV-A, PERV-B and FeLV-B. Values are represented as per cent of infectivities with SD. Small symbols (* and †) indicate significant differences (P<0.05).
ascites were purified using protein A beads, and subsequently used as mAb in this study.

First, we examined whether the mAb KRT1 recognizes PERV-B Env by an indirect immunofluorescence assay (IFA) and flow cytometric analysis. For IFA, HEK293 and HEK293/PERV-B cells were detached by PBS containing 5 mM EDTA and fixed to glass slides with 1% paraformaldehyde at room temperature (RT) for 30 min. After being washed with PBS, the cells were reacted with 10 µg ml⁻¹ of mAb KRT1 and a normal mouse IgG (Sigma) at RT for 45 min, respectively. Then, cells were washed and incubated with a goat anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen) at RT for 45 min. These cells were analysed by fluorescence microscopy (IX72 UV microscope; Olympus). For flow cytometric analysis, HEK293 and HEK293/PERV-B cells were incubated with 10 µg ml⁻¹ of KRT1 or normal mouse IgG on ice for 60 min. From there, the cells were washed and reacted with a goat anti-mouse IgG conjugated with Alexa Fluor 488 on ice for 60 min. The cells were washed with PBS and subsequently analysed using a FACS Calibur (Becton, Dickinson & Company).

Throughout the procedures, cells were treated with 0.2% sodium azide (Nacalai Tesque) to inhibit internalization of the antibodies. As can be seen in Fig. 1(b, c), we detected fluorescence-positive cells in HEK293/PERV-B cells but not in uninfected HEK293 cells, indicating that mAb KRT1 specifically recognizes PERV-B.

Next, to investigate whether the mAb KRT1 possesses neutralization activities, a neutralizing assay was conducted using LacZ pseudotype viruses. LacZ pseudotype viruses, termed LacZ(PERV-B), LacZ(PERV-A) and LacZ(FeLV-B), were prepared from culture supernatants of TELCeB6/FBPERV-B, TELCeB6/FBPERV-A and TELCeB6/FBFeLV-B, respectively. Virus titres were determined in HEK293T cells as described by Sakaguchi et al. (2008) and expressed as f.f.u. ml⁻¹. The titres of the pseudotype viruses were adjusted at approximately 1 x 10³ f.f.u. ml⁻¹ and then subjected to the neutralizing assay. The pseudotype viruses were incubated with 10 µg ml⁻¹ of each antibody [KRT1 and a normal mouse IgG (Sigma)] at 37 °C for 1 h, and then inoculated into HEK293T cells, which were subcultured (1 x 10⁵ cells) in 48-mutiwell plates 1 day before inoculation. Two hours post-inoculation, supernatants were replaced with fresh medium, and the infected cells were incubated for an additional 2 days before X-Gal staining. Assays were performed in triplicate and repeated as four independent experiments. Significant differences (P<0.05) were determined by the Tukey–Kramer multiple comparison test. Although KRT1 did not neutralize LacZ(PERV-A) and LacZ(FeLV-B), it neutralized LacZ(PERV-B) (P<0.05) (Fig. 1d). However, infectivity of LacZ(PERV-B) was not completely diminished at 10 µg KRT1 ml⁻¹, indicating that a higher concentration of the mAb would be needed for complete neutralization.

To determine which region of the Env contains the epitope for KRT1, deletion mutants of PERV-B SU Env were generated. cDNAs for PERV-B SU Env and its deletion mutants were amplified by PCR using the primer sets listed in Supplementary Table S1 (available in JGV Online) and cloned into a pCMV-Tag 4A vector (Agilent Technologies) to add a FLAG tag sequence. Next, the PERV-B SU Env and the deletion mutants tagged with FLAG, termed PBSUFULL-FLAG, PBSUACR-FLAG, PBSUAPR-FLAG, PBSUAVR-FLAG, PBSU1-266-FLAG and PBSU1-250-FLAG (Fig. 2a), were cloned into a pFBSMOSALF vector (Cosset et al., 1995) by an In-Fusion Advantage PCR Cloning kit (TaKaRa) and the resultant plasmids were transfected into TELCeB6 cells. Forty-eight hours post-transfection, TELCeB6 cells were lysed with lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS), and the soluble fractions were collected. Collected samples were subjected to immunoblot analysis using a mouse anti-FLAG mAb M2 (Sigma) and KRT1. Although all the FLAG-tagged proteins were detected by an anti-FLAG mAb (Fig. 2b, d), KRT1 detected PBSUFULL-FLAG, PBSUACR-FLAG and PBSU1-266-FLAG, but not PBSUAPR-FLAG, PBSUAVR-FLAG and PBSU1-250-FLAG (Fig. 2c, e). These data indicated that the epitope for KRT1 resides in the PRR. To determine the epitope of the mAb KRT1 more precisely, we conducted peptide ELISA using 12 overlapping synthetic peptides, corresponding to the amino acid positions 241–275 of PERV-B SU Env. Each peptide, consisting of 13 aa and was continuously shifted by two residues from one peptide to the next. Each peptide, PBSUFULL-HIS (positive control) or BSA (negative control) at 10 nmol per 100 µl per well of peptides was coated on a 96-multiwell plate (Immuno 96 MicroWell Solid Plates; Nunc) and reacted with 50 ng per 100 µl per well of KRT1. Then, the antigen–antibody complexes were detected using an anti-mouse IgG antibody conjugated with HRP (Pierce) and SureBlue Reserve TMB 1-Component Microwell Peroxidase Substrate (KPL). Finally, the enzyme–substrate reaction was stopped with 1 M HCl and the values were measured by scanning OD₄₅₀ using model 680 microplate reader (Bio-Rad Laboratory). Differences were calculated using Tukey–Kramer multiple comparison tests and all differences were considered significant at P<0.05. As shown in Fig. 2(f), KRT1 strongly reacted with peptides harbouring amino acid positions 251–261, indicating that the recognized epitope is ALEPPHNLPVP. Finally, we conducted immunoblot analysis using PERV-A SU tagged with a C-terminal His, termed PASUFULL-HIS (Fig. 2a), to confirm whether the KRT1 distinguishes PERV-B from PERV-A. As a result, PASUFULL-HIS was not detected by KRT1 (Fig. 2g). These data are reasonable given that sequences in the PRR are divergent between PERV subgroups (Fig. 2h) (Le Tissier et al., 1997; Watanabe et al., 2005).

Previously, Lavillette et al. (1998) and Lu & Roth (2003a, b) revealed that MLV PRR was essential to maintain the correct conformation and fusogenicity of Env. However, mAbs against the PRR of ecotropic MLV did not neutralize the virus (Burkhart et al., 2003), indicating that MLV Env was
not affected by binding of these mAbs. On the contrary, mAb KRT1 generated in this study recognized PERV-B PRR and neutralized PERV-B. The binding of KRT1 to PERV-B PRR might have affected the conformation, fusion activity and binding properties of the PERV-B Env.

To date, only two mAbs have been developed against PERV-A, one of which, also recognizes both PERV-A and PERV-B through a highly conserved region among PERV subgroups (Chiang et al., 2007). Our study reveals a novel PERV-B Env-specific mAb that may serve as a useful tool for studying PERV-B infection and help to gain insights into the viral pathogenicity. Moreover, knowledge gained from the use of this antibody may also lead to the development of new protective strategies against PERV-B infection in the context of xenotransplantation.

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