Activation of Porcine Cytomegalovirus, but Not Porcine Lymphotropic Herpesvirus, in Pig-to-Baboon Xenotransplantation

Nicolas J. Mueller,1 Christine Livingston,1 Christoph Knosalla,4 Rolf N. Barth,2 Shin Yamamoto,2 Bernd Gollackner,2 Frank J. M. F. Dor,2 Leo Buhler,2 David H. Sachs,2 Kazuhiko Yamada,2 David K. C. Cooper,2 and Jay A. Fishman1

1Infectious Disease Division and 2Transplantation Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Boston

Tissue-invasive disease due to porcine cytomegalovirus (PCMV) has been demonstrated after pig-to-baboon solid-organ xenotransplantation. Porcine lymphotropic herpesvirus (PLHV)–1 is associated with B cell proliferation and posttransplant lymphoproliferative disorder after allogeneic bone marrow transplantation in swine but has not been observed in pig-to-primate xenotransplantation. Activation of PCMV and PLHV-1 was investigated in 22 pig-to-baboon xenografts by use of quantitative polymerase chain reaction. PCMV was found in all xenografts; increased viral replication occurred in 68% of xenografts during immunosuppression. PLHV-1 was found in 12 xenografts (55%); no increases in viral replication occurred during immunosuppression. Control immunosuppressed swine coinfected with PCMV and PLHV-1 had activation of PCMV but not PLHV-1. PCMV, but not PLHV-1, is activated in solid-organ xenotransplantation.

Herpesviruses, including cytomegalovirus (CMV) and Epstein-Barr virus (EBV), are associated with significant morbidity after solid-organ transplantation [1]. The activation of herpesviruses reflects activation of latent infection by immunosuppression and alloimmune responses to the graft, resulting in invasive disease, rejection of the graft, opportunistic infections, and oncogenesis [1]. Among the γ-herpesviruses, EBV and Kaposi sarcoma–associated herpesvirus (KSHV; also known as human herpesvirus [HHV]–8) are associated with non-Hodgkin lymphomas in immunocompromised hosts [2].

The risk for posttransplant lymphoproliferative disorder (PTLD) is increased by CMV coinfection.

Xenotransplantation of swine tissues has been proposed as a way of alleviating the shortage of human organs for transplantation. Concerns exist regarding the potential spread of novel pathogens from xenograft tissues to recipients [3]. Porcine CMV (PCMV) is activated in pig-to-primate xenografts [4–6], porcine endogenous retroviruses (PERVs) infectious for human cells have been identified in vitro, and porcine lymphotropic herpesvirus (PLHV)–1 and –2 are γ-herpesviruses that are homologous to human EBV and human KSHV [7, 8]. After T cell–depleted, allogeneic stem-cell transplantation, PLHV-1 is up-regulated in association with B cell proliferation and systemic lymphadenopathy progressing to death [8, 9]. No culture systems exist for PLHV-1. Lymphocryptoviruses may lack strict host specificity and could pose a hazard to recipients of PLHV-infected xenografts [10].

PCMV and PLHV-1 infections were investigated in 22 pig-to-baboon xenografts by use of quantitative polymerase chain reaction (PCR). During immunosuppression, activation of PLHV-1 did not occur, whereas activation of PCMV was consistently observed. The differential activation of porcine herpesviruses is consis-
tent with observations in other species; this model provides an opportunity for studies of viral activation and PTLD.

MATERIALS AND METHODS

Animals. Conventionally reared, Large White/Landrace cross-breed pigs transgenic for human decay-accelerating factor (n = 17; Novartis Pharmaceuticals) or Massachusetts General Hospital (MGH) miniature swine (n = 5) were used as donors. Baboons (Papio anubis), (n = 22; 8–20 kg) were purchased from Biological Resources Foundation or Mannheimer Foundation. Animal protocols were approved by the MGH Subcommittee on Research Animal Care.

Pig-to-baboon kidney, thymokidney, thymic lobe, and heart xenotransplants. Procedures and regimens are described in detail elsewhere [11–14]. Two procedures were used in kidney xenotransplantation: transplantation of a kidney without prior manipulation [11] or transplantation of a composite thymokidney created by autologous transplantation of porcine thymic tissue from the native thymus under the renal capsule [13]. In thymic lobe transplantation, a vascularized pig thymic lobe was transplanted into a recipient baboon [12]. Heterotopic heart xenotransplants were performed by anastomosis of the donor aorta to the recipient inferior vena cava [14].

Immunosuppressive regimen and maintenance. Immunosuppression was achieved via thymectomy or thymic irradiation [15], splenectomy, extracorporal adsorption of anti-Gal 3Gal (Gal) antibodies, T cell depletion with antithymocyte globulin (Pharmacia/Ujphohn), cyclophosphamide (Mead Johnson Oncology Products), LoCD2b (rat antiprimate CD2b monoclonal antibody [MAb]; BioTransplant), complement depletion, mycophenolate mofetil, methylprednisolone, and anti-CD154 MAb [11]. Some thymic lobe recipients received tacrolimus.

Baboons were monitored daily. Blood chemistry and hematologic data were obtained daily, and surveillance blood cultures were performed twice weekly. Baboons received prophylaxis with cefazolin sodium (48 h for surgical procedures) and levofloxacin after transplantation. Antimicrobials were adjusted according to culture data or clinical conditions. Baboons were transfused as necessary with irradiated packed red blood cells, platelets, and Gal-depleted fresh frozen plasma from donor baboons. Twelve (54%) recipient baboons received ganciclovir to prevent activation of baboon CMV; ganciclovir does not affect activation of PCMV or PLHV-1 [6].

Control swine. Two MGH miniature swine were immunosuppressed without transplantation. Each underwent splenectomy (day 0) and received mycophenolate mofetil (started day 0, at 110 mg/kg/day to maintain a whole-blood level of 3–7 μg/mL), induction cyclophosphamide (total dose, 100 and 120 mg/kg, respectively), methylprednisolone (2 mg/kg/day, reduced to 0.5 mg/kg/day over the course of 4 weeks), and cyclosporine (15–25 mg/kg/day to maintain blood trough levels of 400–800 ng/mL). The pigs were monitored for 36 days and then euthanatized.

DNA extraction. Tissue specimens were snap-frozen at the time of biopsy. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by use of density gradient sedimentation (Histopaque-1077; Sigma Diagnostics). DNA was isolated by use of the Purgene DNA Isolation Kit (Purgene) using 5–10 mg of tissue. Quantitation of total DNA was performed by use of Hoechst dye fluorescence assay on a DNA fluorometer (Hoefer Scientific Instruments). Results from thymokidney experiments are from kidney xenograft tissue.

Qualitative PCR for PLHV-1 and -2. Published primers and conditions specific for PLHV-1 (170-S/160-AS) and PLHV-2 (280-S/280-AS) were used for conventional PCR [16]. Sequencing of the product confirmed specificity of the primer pairs for PLHV-1 and -2, respectively. The PCR was performed in a GenAmp PCR System 9700 (Perkin Elmer). PCR conditions were as follows: an initial activation step of 10 min at 95°C, 40 cycles of denaturation for 20 s at 94°C, annealing for 20 s at 55°C, extension for 20 s at 72°C, and a final extension of 10 min at 72°C. No cross-reactivity was observed for primer pairs with PCMV. DNA extracted from a lymph node from a pig with PTLD served as a positive control for PLHV-1. As a standard, amplified target DNA was extracted from the gel after conventional PCR and was quantified, to calculate copy numbers in stock solution. A 10-fold dilution was made with water containing salmon sperm DNA, resulting in the equivalent amount of DNA per reaction, compared with total DNA in each specimen (~200 ng/reaction).

Quantitative real-time PCR. Target DNA sequences were quantified by use of real-time PCR using the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer), as described elsewhere [4]. Sequence-specific primers were generated for each gene target (Primer Express software; Perkin-Elmer). Each PCR mix included target DNA, 900 nmol/L primers, 200 nmol/L probe, and TaqMan Universal PCR 2X MasterMix (Applied Biosystems, with passive reference dye ROX; final reaction volume, 50 μL) in a 96-well plate. The PCR conditions were as follows: initial cycle of 2 min at 50°C and 10 min at 95°C, 50 cycles of denaturation for 15 s at 95°C, and annealing/extension for 1 min at 60°C. Target DNA was detected with a linear dynamic range of 10^5–10^9 copies (data not shown). The threshold cycle values of PCR amplification of the standards generated standard curves for quantitation of target DNA.

Measurement of PLHV-1 DNA. Primers and probe specific for PLHV-1 were derived from the PLHV-1 polymerase gene (PLHV-1) [16]: 5′-AAGGTGACATGCAATGCTGTG-3′ (sense), 5′-TGCAATCTTGAGACAGGGCA-3′ (antisense), and
<table>
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<tr>
<th>Xenograft</th>
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<th>Xenograft survival, days</th>
<th>Detection of PLHV-1 by conventional PCR</th>
<th>Activation by real-time PCR</th>
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**NOTE.** PCR, polymerase chain reaction; –, negative; +, positive.

a Activation of PLHV-1 is defined as grafts with \(1 \times 10^1\) PLHV-1 copies/\(\mu\)g of total DNA.

b Activation of PCMV-1 is defined as grafts with \(1 \times 10^4\) PCMV-1 copies/\(\mu\)g of total DNA.

5′-FAM-TGGGTTCACTGTTGTTGATCCTGATG-TAMRA-3′ (probe). Reverse primers and probe were specific for PLHV-1 by BLAST analysis (available at http://www.ncbi.nlm.nih.gov/BLAST/); forward primer matched both PLHV-1 and -2. Sequencing of product amplified by conventional primers confirmed specificity for PLHV-1. No cross-reactivity was demonstrable with PCMV. The quantitative detection limit for this assay was 6 copies. Results are expressed as copy number of PLHV-1 per 1 \(\mu\)g of total DNA (tissue specimens) or copy number per \(1 \times 10^4\) copies of pig MHCC1, equivalent to \(5 \times 10^7\) PBMCs (blood).

**Measurement of PCMV DNA.** Primers and probe specific for PCMV DNA polymerase gene (PCMV) were described elsewhere and showed no cross-reactivity with PLHV-1 [4, 17]; 5′-GTTCTGGGATTTCCGAGGGTTG-3′ (sense), 5′-ACTTCGTCGCCAGCCTCATCTGA-3′ (antisense), and 5′-FAM-CCAGACAGGACAGCAATGGAGCTG-3′ (probe). The quantitative detection limit for this assay was 4 copies. Results are expressed as copy number of PCMV per 1 \(\mu\)g of total DNA (tissue specimens) or copy number per \(1 \times 10^4\) copies of pig MHCC1, equivalent to \(5 \times 10^7\) PBMCs (blood).

**RESULTS**

**Prevalence of PCMV and PLHV-1 in donor pigs.** Levels of PCMV and PLHV-1 in healthy donor pigs were assessed in multiple tissue specimens from source pigs and from healthy control pigs. PCMV DNA copy numbers in these tissue spec-
Figure 1. In control swine receiving immunosuppression comparable to that of baboon xenograft recipients, porcine lymphotropic herpesvirus (PLHV)-1 DNA levels did not increase over time in serial peripheral blood mononuclear cell (PBMC) samples (A). Porcine cytomegalovirus (PCMV) DNA copy nos. increased and remained elevated in the same samples (B). At the time of death, some tissue specimens contained modestly elevated amounts of PLHV-1 DNA, primarily in lymphatic tissue specimens (C). PCMV was activated in all tissue specimens (D). Copy nos. of PLHV-1 and PCMV are expressed per 5 x 10^5 PBMCs or per 1 µg of total DNA. Results of 1 representative pig are shown. *Negative or below quantitative limit of detection for PLHV-1 or PCMV DNA, respectively.

Spleens from 3 (75%) of 4 donor pigs were positive for PLHV-1 by conventional and quantitative PCR (range, 1.9 x 10^1–1.47 x 10^3/µg of total DNA); 1 spleen (25%) was also positive for PLHV-2. PLHV-1 DNA was measured by use of conventional PCR in a variety of tissue specimens from healthy pigs (n = 9 pigs; n = 27 tissue specimens) and was found preferentially in lymphoid tissue specimens (lymph node, salivary gland, thymus, and spleen), compared with other tissue specimens (lung, liver, and kidney). Of these 9 pigs, 7 had at least 1 tissue specimen with PLHV-1, indicating a prevalence of 78% in source pigs. DNA sequencing of amplified PCR products confirmed the presence of both PLHV-1 and -2. Splenic PLHV-1 DNA was not predictive of the presence of PLHV-1 in other organs from the same pig. In one pig with splenic PLHV-1, the heart was negative; a second pig had renal PLHV-1, whereas the spleen was negative. In 2 pigs, the spleen and xenografts (kidney and heart) were positive for PLHV-1.

Activation of PCMV and PLHV in the xenograft. Xenograft biopsy specimens were procured at the time of xenograft removal (7–32 days; table 1). By use of conventional PCR, PLHV-1 was found in 12 xenografts (55%), whereas PLHV-2 was detected in 2 xenografts (9%). PCMV DNA was detected in all xenograft tissue specimens. With xenotransplantation and intensive immunosuppression, no increases were observed in PLHV-1 loads in the 12 xenografts positive for PLHV-1 (<10 copies/200 ng of input DNA). In the same 12 xenografts, 11 (91%) developed increased copy numbers of PCMV DNA (average, 6.87 x 10^3 copies/µg of total DNA; range, 3.18 x 10^3–2.45 x 10^5 copies/µg of total DNA) during immunosuppression. PLHV-1 and PCMV were detected in all 3 types of xenografts (thymic lobe, kidney, and heart) and in PBMCs and splenic tissue specimens; PLHV-2 was found only in renal xenografts and a single spleen. In thymokidney experiments, the level of viral activation was similar in both kidney and thymic tissue.

Activation of PCMV and PLHV in immunosuppressed control pigs. Two healthy pigs with latent PLHV-1 and PCMV
were intensively immunosuppressed with a regimen similar to that used for baboon xenograft recipients. PCMV and PLHV-1 are detected routinely in PBMCs with active viral replication in other tissue specimens (data not shown). No increase in PLHV-1 load was detected in serial porcine PBMC samples (figure 1A; data from 1 representative pig), whereas PCMV DNA increased over time (minimum 1000-fold increase of PCMV DNA copies/5 × 10^3 PBMCs vs. baseline; figure 1B). In multiple tissue specimens from immunosuppressed control pigs, PLHV-1 was minimally elevated only in lymphoid tissue specimens (tonsils, lymph nodes, and thymus) and was below the limits for quantitative detection in other organs (figure 1C). All tissue specimens exhibited high levels of PCMV DNA (figure 1D).

**DISCUSSION**

In swine, 2 herpesviruses have been described: PCMV, a β-herpesvirus, and PLHV (subtypes 1 and 2), a γ-herpesvirus [4–7]. Although PCMV shares pathogenic and clinical characteristics with human CMV [4–6], the biological features of PLHV are largely unknown.

PLHV-1 is associated with a form of PTLD in pigs that is similar to the B cell, non–Hodgkin lymphomas associated with EBV infections in human transplant recipients and in AIDS [2, 8, 9]. As for EBV, the factors responsible for viral activation and the development of lymphoma remain obscure. Partial-sequence data from PLHV-1 contain regions of significant nucleic acid sequence homology with other lymphocryptoviruses (EBV and HHV-8) and some novel sequences [16]. An EBV–open-reading frame (ORF) BALF1h homolog was identified in the PLHV-1 sequence that is reported to be a viral homolog (v-bcl-2) of the mammalian bcl-2 oncogene, a functional apoptosis inhibitor [8]. PLHV also carries a homolog of EBV-ORF BZLF2h that has been described as encoding a glycoprotein required for the entry of EBV into B cells via HLA-DR. PLHV-1 is detected primarily in lymphoid tissues [7]. Systemic increases in PLHV-1 precede B lymphocyte proliferation after T cell–depleted porcine bone marrow allograft transplantation [9]. As in other models of PTLD, diminished T cell surveillance, allogeneic mismatch, B cell infection by PLHV-1, and other unknown factors are associated with development of lymphoma. The roles of PCMV or PERV in the pathogenesis of PTLD are not known.

In this series of pig-to-baboon xenografts, activation of PCMV (68%) was not uniform. The majority (55%) of the organ xenografts and animals (3 of 4 normal spleens) also carried latent PLHV-1, although some further PLHV infections may have been below the detection limits of our assays [7]. Despite immunosuppression and xenogeneic immune responses, neither porcine xenografts carrying PLHV-1 nor those that were negative at baseline developed activation of PLHV-1. In contrast, in pigs with B cell PTLD, PCMV replication was not significantly increased in pigs demonstrating 100–1000-fold increases in PLHV-1 loads. This is consistent with the absence of significant numbers of target cells (i.e., B cells) to support the replication of PLHV-1 in solid-organ tissue xenografts. Although mouse models may suggest a role for donor-derived T cells in the development of PTLD, host T cell depletion and the allogeneic immune response are critical for the development of PTLD in the allogeneic stem–cell transplantation model [18]. These differences in activation of porcine herpesvirus are consistent with observations made in other species [18]. The long-term consequences of the presence of PLHV-1 in xenografts are unknown. The unique factors associated with the activation of PLHV-1 may have implications for the pathogenesis of EBV or KSHV-associated tumors and merit further study.

**References**