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RAPID COMMUNICATION

Productive Infection of Human Primary Cells and Cell Lines with Porcine Endogenous Retroviruses

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Porcine endogenous retroviruses (PERVs) infect human cells *in vitro* and therefore represent a risk for xenotransplantation. However, first clinical transplantations of pig cells into humans or *ex vivo* perfusions did not result in transmission of PERVs. On the other hand, recent experiments with SCID mice demonstrated infections with PERV *in vivo.* In order to define and characterize human target cells, we studied numerous primary human cells and cell lines. Infection with PERVs was shown for human peripheral blood mononuclear cells, primary endothelial cells, and primary aortic smooth muscle cells as well as lymphocytic, monocytic, and epithelial cell lines.

Introduction. Progress in transplantation medicine and the shortage of human organs is the driving force in the search of alternative methods to allotransplantation. One of the most promising applications is xenotransplantation. Pigs are favored as potential donors of cells, tissues, and organs in comparison with nonhuman primates because of a lower contamination with microorganisms, the availability of transgenic animals, and lower costs (*1*). Since most micro-organisms of the pig with the potential to cause a zoonosis can be eliminated by specified pathogen-free containment, the most pronounced risk comes from unknown organisms and from porcine endogenous retroviruses (PERVs) (*4*). Different types of PERV are present in the genome of all pig strains and at least PERV-A and PERV-B are able to infect human cells *in vitro* (*6, 8, 10, 11, 14, 16, 21, 22*). Investigations of human recipients who had short-term contact with porcine cells or tissues and of nonhuman primates with pig cell or organ transplantation showed no evidence of PERV infection (*9, 12, 15, 18, 20*). In contrast to these results infection of SCID mice with PERV after porcine islet xenotransplantation has been recently described (*3, 5*), showing that *trans*-species transmission of PERV *in vivo* is possible. In order to evaluate the potential risk posed by PERVs during the future clinical use of porcine xenografts, understanding the mechanism of virus transmission and the nature and range of human target cells is essential. We show that all primary human cells and cell lines tested could be infected (and except one, all of them produce virus particles), indicating that a wide range of human cells carry the still unknown receptors for PERVs.

Results and Discussion. PERV-NIH, a PERV-A/C recombinant produced by human 293 cells (kindly provided by C. Wilson, FDA, Washington; *23*) was serially passaged on the human kidney cell line 293. After each passage the virus titer increased and the time to detect infection decreased as has also been shown for PERV from pig PK-15 cells, passaged on human 293 cells (*16*). Passage 5 of PERV-NIH was used for the infection experiments with all human cells and cell lines listed in Table 1. Infection was evaluated by PCR using primers specific for PERV *gag, env,* and *pol.* Positive PCR amplification was seen in all cases starting with day 10 postinfection showing the presence of proviral DNA in infected cells (Table 1, Fig. 1). In addition, cell-free supernatants from infected cells were screened for RT activity for the detection of productive infection. The human kidney cell line 293, primary pulmonary artery endothelial cells (HPAEC), primary aortic endothelial cells (HAEC), and primary human peripheral blood mononuclear cells (PBMCs) showed RT activity already 1 week postinfection. The human lymphocytic cell lines C8166, THP-1, and WIL2.NS.6TG were positive for type C retroviral-specific RT activity only 3–5 weeks postinfection. However, the primary coronary artery smooth muscle cells (HCASMC) showed, despite detection of provirus, no RT activity during the time of cultivation. Because of the limited life span of these primary cells they could not be studied later on. To investigate the productive infection of the target cells in more detail, virus was pelleted from cell

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TABLE 1

Infection of Human Cells

Cells	Tissue	Detection of PERV			
		PCR	mU RT/ml[a]	RT-PCR	IPA
PBMC	Blood	+	12 ± 3	+	n.t.
C8166	T cell line	+	10 ± 2	+	+
THP-1	Monocyte cell line	+	19 ± 2	+	+
WIL2.NS.6TG	Spleen cell line	+	37 ± 5	+	+
HPAEC	Primary pulmonary artery endothelial cells	+	15 ± 1	n.t.	n.t.
HAEC	Primary aortic endothelial cells	+	17 ± 3	n.t.	n.t.
HCASMC	Primary coronary artery smooth muscle cells	+	—	n.t.	n.t.
293	Kidney cell line	+	530 ± 12	+	+

Note. n.t., not tested.

[a] Mean ± SD of triplicate measurement in one experiment. Some experiments were performed up to 10 times showing similar data.

culture supernatants and viral RNA was isolated. In a RT-PCR using primers specific for PERV *pol,* RNA from viral particles derived from PERV-infected 293, C8166, THP-1, and WIL2.NS.6TG cells showed positive amplicons (Table 1, Fig. 2). In addition, the human lymphocytic cell line C8166, the monocytic cell line THP-1, and the spleen cell line WIL2.NS.6TG as well as the human kidney cell line 293 were also tested for the expression of viral proteins by an immunoperoxidase assay (IPA) using a PERV-specific antiserum against the recombinant envelope protein rp15E. In all cases cells showed expression of p15E indicating, apart from integration of proviral DNA, the expression of viral proteins and supporting production of virus particles. The ability to infect the chosen primary human cells as well as human PBMCs, lymphocytic cells, and monocytic cells productively with PERV has severe implications since circulating immune cells come into close contact with the xenotransplant and may transmit the virus to other human cell compartments. In that context, productive infection of C8166 cells with virus produced by PERV-infected C8166 cells (10 ± 2 mU RT activity/ml) was shown. Virus production was demonstrated by measuring RT activity in the supernatant (9 ± 1 mU RT activity/ml), indicating transmission and replication competence of PERV on this human T cell line. But replication has been to a smaller extent in comparison to uninfected human 293 cells inoculated with the same amount of PERV from infected C8166 cells in parallel. Similar differences were observed with PERV produced from the productively infected mink lung cell line Mv1Lu. Virus produced from PERV-infected Mv1Lu cells more easily infected 293 cells in comparison to Mv1Lu cells (V. Specke *et al.,* in preparation). It is noteworthy to stress that the human kidney cell line 293 showed the highest sensitivity for PERV infection among all human cells tested here (Table 1) and the amount of virus produced by 293 cells is much higher in comparison with pig kidney cell lines producing PERV

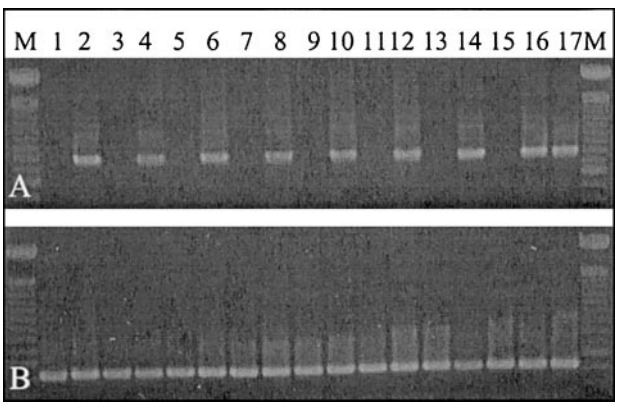

FIG. 1. Detection of PERV in infected human cells by PCR. Primers specific for (A) PERV *pol* (expected amplicon 817 bp) and (B) human β-actin (expected amplicon 528 bp) were used: lanes 1, uninfected HPAEC; 2, infected HPAEC; 3, uninfected HAEC; 4, infected HAEC; 5, uninfected HCASMC; 6, infected HCASMC; 7, uninfected C8166; 8, infected C8166; 9, uninfected THP-1; 10, infected THP-1; 11, uninfected WIL2.NS.6TG; 12, infected WIL2.NS.6TG; 13, uninfected PBMC; 14, infected PBMC; 15, uninfected 293; 16, infected 293; 17, 293-NIH/passage 5; M, 100 bp ladder.

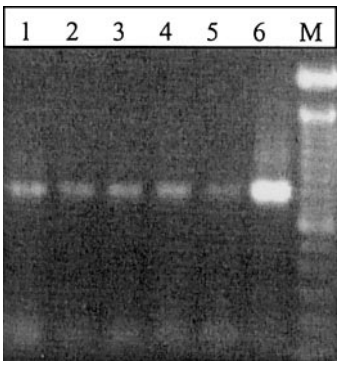

FIG. 2. One-step RT-PCR with RNA from pelleted virus produced by infected human cells using primers specific for PERV *pol* (expected amplicon 817 bp): lanes 1, 293; 2, C8166; 3, THP-1; 4, WIL2.NS.6TG; 5, PBMC; 6, 293-NIH/passage 5; M, 100 bp ladder.

TABLE 2

Comparison of PERV Production by Porcine and Human Cells

Species	Cell line	mU RT/ml[a]
Pig	PBMC[b]	5 ± 2
	PK-15	70 ± 10
Human	293-NIH/5	530 ± 12

[a] Mean ± SD of triplicate measurement in one experiment. Some experiments were performed up to 10 times showing similar data.
[b] Mitogen stimulated.

continuously (Table 2). In addition, all (except one) human primary cells and cell lines tested released PERV on higher levels than primary pig PBMCs after mitogen stimulation (Tables 1 and 2). This suggests that PERVs which are released from porcine cells at a very low titer are able to increase their replication competence after infection of human cells.

Taken together, our data and recent publications on the host range of PERV (*6, 7, 10, 11, 14, 16, 21, 22*) show that human cells are very permissive for PERVs. Furthermore, we showed for the first time (i) productive infection of human PBMCs; (ii) infection of primary muscle cells; (iii) productive infection of human lymphocytic, monocytic, and spleen cell lines with PERV; and (iv) productive transmission of PERV produced from the human T cell line C8166 to uninfected C8166 cells. These data strongly emphasize the need for further research in the field of PERV transmission before commencing with xenotransplantation in humans. For that reason we started, in addition to the attempts to establish a small animal model (*16*), an infection study with nonhuman primates to obtain further information on the infection potential of PERV *in vivo*.

Materials and Methods. Virus production. PERV-NIH (kindly provided by C. Wilson, FDA, Washington, DC; PERV-NIH, 3rd passage) (*23*) was passaged on human 293 kidney cells and passage 5 was used for infections that were carried out using cell-free cell culture supernatants. For comparison, PERV produced by the pig kidney cell line PK-15 (ATCC CCL 33, U.S.A.), and PERV released from PBMCs isolated from the blood of healthy outbred Yucatan micropigs (Charles River, Germany) as described (*19*), cultured in RPMI 1640 with 10% FCS, stimulated with 72 μg/ml phytohemagglutinin (Abbott Murex, Germany) for 5 days in the presence of 100 IU/ml IL-2 (EuroCetus GmbH, Germany) were studied.

Cells Used for Infection. The human T cell line C8166 (ECACC, 88051601), the human monocytic cell line THP-1 (ATCC, TIB-202), the uninfected human kidney cell line 293 (also kindly provided from C. Wilson, FDA, Washington, DC), the human spleen cell line WIL2.NS.6TG (ECACC, 93031001), human primary pulmonary artery endothelial cells, human aortic endothelial cells, and human coronary artery smooth muscle cells (all three Cascade Biologics, Inc., Portland, OR) were cultured according to the directions of the contributors. Human PBMCs were isolated from healthy blood donors as described elsewhere (*19*) and grown in RPMI 1640 with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies GmbH, Germany). All cells were inoculated with a standard virus dose of 1×10^5 $TCID_{50}$/ml for 24 h in the presence of 8 μg/ml Polybrene (Sigma, Deisenhofen, Germany).

PCR. PCR was performed as described previously (*16*) using primers specific for PERV *gag* (forward, 5′-GCG ACC CAC GCA GTT GCA TA; reverse, 5′-CAG TTC CTT GCC CAG TGT CCT T; and forward, 5′-TGA TCT AGT GAG AGA GGC AGA G; reverse, 5′-CGC ACA CTG GTC CTT GTC G (*12*)), *env* of PERV-A (forward, 5′-TGG AAA GAT TGG CAA CAG CG; reverse, 5′-AGT GAT GTT AGG CTC AGT GG (*6*)), and *env* of PERV-B (forward, 5′-TTC TCC TTT GTC AAT TCC GG; reverse, 5′-TAC TTT ATC GGG TCC CAC TG (*6*)) as well as for *pol* of PERV (forward, 5′-TTG ACT TGG GAG TGG GAC GGG TAA C; reverse, 5′-GAG GGT CAC CTG AGG GTG TTG (*2*)). Amplification was carried out using standard PCR conditions on a Biozym cycler (Biozym Diagnostic, Oldendorf, Germany): 1 initial cycle of 10 min denaturation at 95°C; 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 58°C, 1 min elongation at 72°C; and 1 final cycle of 7 min elongation at 72°C.

RT-PCR. For RT-PCR viral RNA from supernatants of cultured cells was isolated. Cells were removed from supernatants by centrifugation at 200 *g* for 10 min. Thereafter cell debris were removed by centrifugation at 3500 *g* for 10 min and an additional centrifugation step at 10,000 rpm (SW 28; Beckmann, Germany) for 10 min. Virus was pelleted by ultracentrifugation (28,000 rpm for 3 h, SW 28) and viral RNA was isolated using the viral RNA isolation kit from Qiagen GmbH (Germany). RNA was reverse transcribed using a one-step RT-PCR kit (Life Technologies GmbH) and cDNA was screened for detection of PERV using PCR carried out with PERV-specific primers as described above.

RT Assay. For detection of productive infection of target cells with PERV a commercial reverse transcriptase assay (Cavidi Tech, Uppsala, Sweden) was used.

Immune Peroxidase Assay. Virus protein expression was analyzed using an IPA performed as described (*17*). Briefly, cells were trypsinized and seeded in a six-well plate (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 over night at −20°C. Cells were treated with 2% fat-free milk powder (Marvel, UK) in PBS for 1 h to block nonspecific

antibody binding. After blocking, the cells were incubated for 1 h with PERV-specific antiserum against recombinant p15E (diluted 1:100 in blocking solution). After four washes with PBS, protein G labeled 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 3-amino-9-ethylcarbazole (Sigma) was added.

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