Reduced Sensitivity to Human Serum Inactivation of Enveloped Viruses Produced by Pig Cells Transgenic for Human CD55 or Deficient for the Galactosyl- α (1-3) Galactosyl Epitope

Saema Magre,¹ Yasuhiro Takeuchi,^{1*} Gillian Langford,² Andrew Richards,² Clive Patience,³ and Robin Weiss¹

Department of Immunology and Molecular Pathology, University College London, London,¹ and Imutran Ltd. (a Novartis Pharma A.G. Company), Cambridge,² United Kingdom, and Immerge BioTherapeutics Inc., Cambridge, Massachusetts³

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Complement activation mediated by the major xenogeneic epitope in the pig, galactosyl- α (1-3) galactosyl sugar structure (α -Gal), and human natural antibodies could cause hyperacute rejection (HAR) in pig-to-human xenotransplantation. The same reaction on viruses bearing α -Gal may serve as a barrier to zoonotic infection. Expressing human complement regulatory proteins or knocking out α -Gal epitopes in pig in order to overcome HAR may therefore pose an increased risk in xenotransplantation with regard to zoonosis. We investigated whether amphotropic murine leukemia virus, porcine endogenous retrovirus, and vesicular stomatitis virus (VSV) budding from primary transgenic pig aortic endothelial (TgPAE) cells expressing human CD55 (hCD55 or hDAF) was protected from human-complement-mediated inactivation. VSV propagated through the ST-IOWA pig cell line, in which α -galactosyl-transferase genes were disrupted (Gal null), was also tested for sensitivity to human complement. The TgPAE cells were positive for hCD55, and all pig cells except the Gal-null ST-IOWA expressed α -Gal epitopes. Through antibody binding, we were able to demonstrate the incorporation of hCD55 onto VSV particles. Viruses harvested from TgPAE cells were relatively resistant to complement-mediated inactivation by the three sources of human sera tested. Additionally, VSV from Gal-null pig cells was resistant to human complement inactivation. Such protection of enveloped viruses may increase the risk of zoonosis from pigs genetically modified for pig-to-human xenotransplantation.

The species disparity between pigs and humans, resulting in the immunological rejection of porcine organs in human xenograft recipients, is a major hurdle to the success of xenotransplantation. Hyperacute rejection (HAR) is the most rapid rejection, occurring within minutes of exposure of galactosyl- α (1-3) galactosyl (α -Gal)-positive pig organs to human serum. It is primarily mediated by activation of the human complement cascade following the interaction of α -Gal epitopes on pig cells and anti- α -Gal xenoreactive antibodies naturally present in humans and in Old World primate serum (1, 15, 41). Due to its nonspecific nature, the complement system is regulated by a family of complement-regulatory proteins (CRPs) thought to function in a species-specific manner (23). The suggestion that human CRPs (hCRP) expressed on pig organs may prevent HAR has led to the development of pigs transgenic for hCRPs, including decay-accelerating factor (DAF; CD55), membrane cofactor protein (CD46), and membrane inhibitor of reactive lysis (CD59) (3, 14, 18, 54). Organs from such transgenic pigs have shown improved survival when transplanted into nonhuman primates (20, 22, 29). More recently, animals lacking the α -Gal epitope have been created by knocking out the α -galactosyl transferase gene (*GGT1*) (32). It is expected that organs from such animals will escape anti- α -Gal-mediated xenoreactive immune responses.

Zoonosis, the inadvertent transfer of pathogens from one species to another, such as pigs to humans, presents another potential obstacle associated with xenotransplantation. Although the phylogenetic distance between pigs and humans may reduce the chances of cross-species infections, pigs harbor several pathogens infectious to humans (20). Known pathogens can be excluded from source pig herds by specific-pathogen-free breeding of herds. However, unknown, endogenous, or asymptomatic microbes may provide a significant risk of infection. Such agents include the gammaretrovirus porcine endogenous retroviruses (PERV) and a number of other recently discovered porcine viruses (20). Although PERV are present in multiple copy numbers in the pig genome and can infect human cells in vitro (21, 31, 51), retrospective studies on xenograft recipients have found no evidence for infection of PERV in humans (11, 16, 28, 30, 33).

Enveloped viruses are known to incorporate host cell membrane proteins as they bud from a producer cell (27). Viruses budding from α -Gal-positive animal cells incorporate α -Gal epitopes on their surface. These epitopes can bind anti- α -Gal antibodies in human serum, resulting in activation of the complement cascade, leading to virus inactivation. Thus, humans are “protected” from animal viruses (40, 48, 49). Viruses budding from α -Gal-negative human cells lack α -Gal epitopes and can also acquire additional resistance to human complement by incorporating hCRPs into their envelopes (24, 42, 45). PERV released from porcine cells incorporate the α -Gal epitope into their envelopes and undergo lysis in human serum (31). In vitro experiments have demonstrated that xenogeneic

* Corresponding author. Mailing address: Wohl Virion Centre, Windeyer Institute of Medical Sciences, University College London, 46 Cleveland St., London W1T 4JF, United Kingdom. Phone: 44 20 7679 9569. Fax: 44 20 7679 9555. E-mail: y.takeuchi@ucl.ac.uk.

cells expressing human CD46, CD55, and CD59 are protected from lysis in human serum, e.g., porcine endothelial cells (4, 7, 25). Consequently, viruses budding from such transgenic cells are also likely to incorporate hCRPs into their envelopes.

The sensitivity of PERV produced from a porcine pig testis cell line (ST-IOWA) expressing the transfecting hCRP CD59 cDNA to inactivation by human serum has recently been analyzed. PERV were shown to incorporate CD59 into their envelopes, which subsequently protected the virions from complement-mediated lysis as measured by a decrease in reverse transcriptase enzyme release. However, incorporation of CD59 did not appear to affect PERV sensitivity to inactivation by human serum at high serum concentrations (46).

In this report, we examine the effect of human complement on three enveloped viruses, two gammaretroviruses and a rhabdovirus, generated from six different batches of primary porcine aortic endothelial (PAE) cells, three of which are transgenic for human CD55 (TgPAE). The two gammaretroviruses, amphotropic murine leukemia virus (MLV-A), a high-titer virus, and PERV subgroup A (PERV-A), an endogenous retrovirus in pigs, and vesicular stomatitis virus (VSV), a rhabdovirus pig pathogen, were passaged through TgPAE and PAE cells. Viruses were tested for their sensitivities to complement-mediated inactivation by infection assays and for VSV incorporation of human CD55 (hCD55) into virions.

MATERIALS AND METHODS

Cell culture. PAE cells were harvested from three outbred Large White CD55-transgenic pigs (TgPAE A, B, and C), as well as three nontransgenic control pigs (PAE D, E, and F), as previously described (5). Briefly, aortas, surgically removed from heparinized and anesthetized piglets, were rinsed three times with ice-cold Hanks' buffered saline containing penicillin (300 U/ml), streptomycin (300 µg/ml), and gentamicin (150 µg/ml). Aortas were sectioned longitudinally, and the endothelial layer was scraped from the surface with a scalpel blade and then rinsed off into a 0.01% solution of collagenase in Dulbecco's modified Eagle's medium (DMEM). The cell layer was digested by swirling in a 37°C water bath. Ten milliliters of DMEM plus 15% fetal bovine serum was added, and the suspension was centrifuged at 400 × g for 5 min. PAE cells were cultured in DMEM with Glutamax (Gibco BRL) supplemented with 15% heat-inactivated fetal calf serum (FCS) (Helena Biosciences), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco BRL), on 1% gelatin (Sigma)-coated T25 flasks. Cells were passaged every 3 to 5 days at a ratio of 1:5 up to passages 17 to 20. Most assays and experiments were done when the cells were between passages 10 and 17.

ST-IOWA (pig testis) Gal-null and wild-type (37), human HeLa (cervical carcinoma), 293 (human embryonic kidney), TE671 (rhabdomyosarcoma), Mv-1-Lu (mink lung), and NIH 3T3 (mouse embryonic fibroblast) cell lines were cultured in DMEM supplemented with 10% FCS, penicillin, and streptomycin. Cells were grown at 37°C, 5% CO₂, in a humidified atmosphere.

Viruses. The following viruses were used for infectivity studies. (i) For replication-competent MLV-A pseudotypes, TgPAE, PAE, and HeLa cells were first transduced with the retroviral MFGnslacZ vector, by helper-free gibbon ape leukemia enveloped virus (36), and then infected with replication-competent MLV-A as described previously (47). (ii) For replication-competent PERV, irradiated 293 cells infected with 14/220 PERV-A (13, 26) were cocultured with TgPAE (A and B), PAE (E), and HeLa cells. Three weeks later, cell supernatant was harvested, filtered (pore size, 0.45 µm), and concentrated by centrifugation at 3,500 × g for 7 to 10 min in Ultrafree-4 centrifugal filter units (Millipore) before use in infection assays. (iii) For replication-competent VSV (Indiana serotype), stocks were generated in Mv-1-Lu (48) and used at a multiplicity of infection of 0.1 to infect TgPAE (A and C), PAE (D and F), ST-IOWA (wild type), ST-IOWA (Gal null), and HeLa cells. Virus was harvested after 24 h. Viruses used in the complement infection assays were harvested in serum-free OptiMEM (Gibco BRL), filtered (pore size, 0.45 µm; Sartorius), divided into aliquots, and stored at -80°C until required, with the exception of PERV, which was used without freezing.

X-irradiated cell coculture. 293/PERV-A (14/220) producer cells were detached with trypsin-versene (Gibco BRL), pelleted at 500 × g for 10 min, resuspended in 1 ml of culture medium, and X irradiated with 100 Gy (at 5 Gy/min). Cells were then resuspended at a ratio of 4 × 10⁵ X-irradiated producer cells mixed with 4 × 10⁴ uninfected TgPAE (A and C), PAE (D), and HeLa target cells in six-well plates (31). After passage for 3 weeks, when X-irradiated producer cells had disappeared, cells were ready for virus harvesting.

Flow cytometry. Cells were washed twice in phosphate-buffered saline (PBS), detached in 5 ml of 0.02% EDTA-PBS (CD55 staining), and washed with 5 ml of DMEM. For each test, 10⁵ cells were washed three times in ice-cold PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS/BA). Samples were then incubated on ice with either primary mouse anti-human CD55 antibody (BRIC110) or mouse anti-human CD46 antibody (J4-48) from Cymbus Bioscience Ltd., diluted to 1:20 in PBS/BA, for 1 h. After three washes in PBS/BA, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch), diluted to 1:200 in PBS/BA and incubated on ice for 1 h. For α-Gal expression, cells were detached by a cell scraper, washed, resuspended, and stained in a single 1-h incubation with FITC-conjugated *Bandeiraea simplicifolia* isolectin (IB-4) (Sigma) at 10 µg/ml in PBS/BA on ice, as this lectin is specific for the terminal α-Gal sugar. All samples were then washed twice in PBS/BA and once in PBS before being resuspended and fixed in 250 µl of PBS supplemented with 2% formaldehyde (49). Fluorescence-activated cell sorter (FACS) analysis was done with a Becton-Dickinson FACSscan using Cell Quest software. Histograms were generated by using WinMDI software by Joseph Trotter. Expression levels were calculated from the mean fluorescent intensity (MFI) shifts given by the histogram statistics.

Virus binding assay. One hundred microliters each of VSV produced from TgPAE, PAE, and HeLa cells (TgPAE/VSV, PAE/VSV, and HeLa/VSV), at an approximate titer of 10⁸ infectious units/ml, incubated with 1 or 5 µg of monoclonal antibodies, was made up to a total volume of 250 µl in PBS with 3% bovine serum albumin (PBS/BSA). Monoclonal antibodies used were three anti-human CD55 antibodies, anti-DAF (Wako Junyaku, Osaka, catalogue number 016-11951), BRIC 216, and BRIC 110 (Cymbus Bioscience Ltd); an anti-human CD46 antibody, J4-48; and an anti-human CD59 antibody, BRA-10G (Cymbus Bioscience Ltd). The mixture was incubated overnight at 4°C. Four hundred microliters of OMNISORB cells (Calbiochem) were washed once in PBS. The pellet was resuspended in 800 µl of PBS/BSA and incubated for 1 h at room temperature while spinning on a wheel. The cells were then centrifuged at 17,000 × g for 1 h, and the pellet was resuspended in 400 µl of PBS/BSA. Fifty microliters of washed cells were added to 200 µl of the virus-antibody mix and incubated with gentle mixing at room temperature for 30 min. Samples were centrifuged at 3,500 × g for 30 min and washed twice, first in PBS/BSA and then in DMEM-10% FCS. The pellet was finally resuspended in 200 µl of DMEM-10% FCS, and VSV infections were carried out as described below. VSV preparation without OMNISORB treatment was also titrated to measure the input titer. Virus binding is expressed as the percentage of pulled-down titer relative to the input titer as infectious units.

Serum sources. Human serum was collected from three healthy volunteers (designated here as sera 1, 2, and 3). Heat inactivation was carried out at 56°C for 1 h. A twofold dilution series of fresh human serum was made in heat-inactivated human serum, such that only the heat-labile complement components were diluted and all other serum components remained unchanged.

Infections. MLV-A infections were carried out as previously described (47). Target TE671 cells, seeded at 3 × 10⁴ cells/well in 24-well plates, were incubated overnight at 37°C. Forty microliters of neat MLV-A virus mixed with an equal volume of fresh human serum, or fresh human serum diluted in heat-inactivated human serum, in 20 mM HEPES buffer (pH 7), was incubated in a 37°C water bath for 1 h. The virus-serum mixture was then diluted in 1 ml of culture medium with 8 µg of hexadimethrine bromide (polybrene)/ml, and a 10-fold dilution was made of this mixture. These dilutions were used to inoculate target cells. After 3 h, the virus inoculum was removed and replaced with 1 ml of normal culture medium. Three days later, cells were fixed and stained for β-galactosidase (LacZ) expression as described previously (47). Titers of the LacZ pseudotype were determined by counting the number of blue cell clusters, each cluster being attributed to a single infection. Titers were expressed as LacZ infectious units per milliliter of virus supernatant.

PERV was incubated with complement as described above (26 µl of virus with 26 µl of serum diluted to various concentrations using a heat-inactivated aliquot of the same serum as a diluent). The virus-serum mixture was then diluted in 52 µl of normal medium containing 8 µg of polybrene/ml. One hundred microliters of this mix was used to infect 293 target cells, plated at a 2 × 10⁵/ml density, in a 96-well plate. Medium was replaced with 150 µl of supplemented DMEM the

FIG. 1. hCD55 and α -Gal expression on porcine cells. (a) Expression of CD55 was analyzed by FACS on both transgenic (TgPAE) and nontransgenic (PAE) pig cells. Staining by anti-hCD55 (shaded histogram), no primary antibody (negative control; solid line), or anti-hCD46 (dotted line, similar profile to that of the no-antibody control) followed by FITC-labeled secondary anti-mouse immunoglobulin antibodies (upper panels) is shown. Staining for α -Gal was a one-step incubation; hence, -lectin on the histogram represents cells only, and +lectin represents cells incubated with IB-4 lectin conjugated to FITC (lower panels). (b) α -Gal expression on ST-IOWA wild-type and ST-IOWA Gal-null (-/-) cells. Expression of the α -Gal antigen was analyzed by FACS, using the IB-4 lectin. A rightward shift of the histogram in the presence of lectin (+lectin) compared to the histogram generated in the absence of lectin (-lectin) indicates expression of the α -Gal antigen. The level of expression was determined using the MFI shift, generated by the Cell Quest FACSscan software. The MFI shift was calculated by determining test MFI and subtracting that of negative controls (no primary antibodies for hCD55 and hCD46 expression and no lectin for α -Gal expression), and all the mean shifts are shown in Table 1.

next day. Three days after infection the cells were fixed in 1:1 acetone-methanol, immunostained with PERV antcapsid primary antibody followed by goat anti-rabbit secondary antibody conjugated to alkaline phosphatase, and then detected by using 4-nitroblue tetrazolium chloride-5-bromo-4-chromo-3-indolylphosphate (NBT/BCIP) ready-to-use tablets (Roche) as described previously (2).

For VSV infections, 100 μ l of Mv-1-Lu cells were plated at 8×10^4 /ml in 96-well plates. Tenfold dilutions, from 10^{-1} to 10^{-12} , of VSV were made in DMEM. One hundred microliters of each virus dilution was used to infect cells. These were incubated at 37°C for 5 days, after which dead and live wells were scored and the titer of VSV was determined as the 50% tissue culture infective dose (TCID₅₀).

RESULTS

hCD55 and α -Gal expression on aortic endothelial cells from hCD55-transgenic pigs. PAE cells were obtained from three human CD55 (hCD55)-transgenic pigs (TgPAE A, B, and C) as well as three nontransgenic control pigs (PAE D, E, and F). These porcine cells, as well as human and mouse cells,

were stained with antibodies or a lectin to examine levels of hCD55 and α -Gal antigen expression. The increase in the MFI (MFI shift) in the staining in the presence of primary antibody or lectin relative to that in their absence was determined by FACS analysis. Representative FACS histograms are shown in Fig. 1a for TgPAE A and PAE D cells. All TgPAE cells expressed hCD55 at similar levels, with mean shifts ranging from 25 to 30, while none of the PAE cells expressed hCD55 (Table 1). Neither TgPAE nor PAE cells reacted with anti-hCD46 as expected (Fig. 1a) (no MFI shift). HeLa cells showed a significant shift for both hCD55 (MFI shift, 437) and hCD46 (MFI shift, 63) expression, while no shift was observed for mouse NIH 3T3 cells (a negative control) with anti-hCD55 antibody. High levels of α -Gal expression were detected on NIH 3T3 cells but not on HeLa cells, as expected (Table 1, footnote f). Both TgPAE and PAE cells expressed α -Gal, al-

TABLE 1. Summary of virus sensitivity and cell surface molecule expression

Cell line	IS ₉₀ ^a (%)			Expression (mean shift)	
	MLV-A ^b	PERV-A 14/220 ^c	VSV ^d	CD55 ^e	α-Gal ^f
TgPAE A	9.51	20.7	16.1	28.48	75.52
TgPAE B	4.85	>50	NT	25.56	117.28
TgPAE C	12.8	NT	38.2	30.09	90.60
PAE D	0.45	4.99	10.2	0.12	56.17
PAE E	2.69	NT	NT	-0.16	28.35
PAE F	0.56	NT	5.18	-0.13	30.32

^a IS₉₀ values were calculated using a standard formula, $\log IS_{90} = \log \text{conc HPP} - [(HPP - 10)/(HPP - LPP) \times \log d]$, where HPP is the highest % protection closest to but higher than 10%, LPP is the lowest % protection closest to but lower than 10%, conc HPP is the serum dilution (%) at which HPP was observed, and d is the dilution factor.

^b MLV-A complement sensitivity assays using human serum 2.

^c PERV complement sensitivity assays using human sera 1 and 3. NT, not tested.

^d VSV complement sensitivity assays using human serum 3.

^e HeLa cells were tested as the positive control for CD55 expression and showed a MFI shift of 437.76 compared to HeLa cells stained in the absence of primary antibody. CD55-negative NIH 3T3 cells showed no shift compared with the histogram generated in the absence of primary antibody. TgPAE cells all showed a significant mean shift when fluorescence in the presence of primary antibody was compared to that without primary antibody (negative control). As expected, PAE cells showed no significant mean shift when comparing fluorescence with primary antibody to that without primary antibody.

^f NIH 3T3 cells were tested as the positive control for α-Gal expression and showed a mean shift of 101.20. α-Gal-negative HeLa cells showed no shift.

though PAE cells appeared to express lower levels of the epitope than TgPAE cells (Table 1). As expected wild-type ST-IOWA cells expressed α-Gal while ST-IOWA Gal-null cells did not (Fig. 1b).

Preparation of MLV-A, VSV, and PERV particles. MLV-A, VSV, and PERV particles, which can be easily titrated for infectivity, were produced from TgPAE, PAE, and HeLa cells. VSV was also produced from ST-IOWA and ST-IOWA Gal-null cells. HeLa cells, which lack the α-Gal epitope and contain hCRPs, including hCD55, provide virus resistant to inactivation by human serum. Titers of a replication-competent MLV-A LacZ pseudotype ranged from 10³ to 10⁴ infectious units/ml. Titers of VSV based on cytopathic effect (49) ranged between 10⁸ and 10⁹ PFU/ml. PERV-A (14/220) (12, 26) was titrated by an in situ focus-forming immunostaining assay (2), with titers of 10⁴ from virus harvested from HeLa cells and between 10² and 10³ from virus harvested from either PAE or TgPAE cells.

hCD55 incorporation onto virus harvested from transgenic pig cells. We were unable to detect hCD55 incorporation onto virus particles released from TgPAE cells by either Western blotting or particle immunofluorescence staining (34), using several anti-hCD55 monoclonal antibodies, suggesting that hCD55 was not incorporated onto particles or that the level of hCD55 incorporation was low. However, a virus pull-down assay allowed us to demonstrate incorporation of hCD55 on VSV particles. VSV harvested from TgPAE, PAE, and HeLa was incubated with antibodies to hCD55 and protein G-expressing bacterial cells. Titer of virus bound to the cell was measured by plating the suspension of bacterial cells after centrifugation and extensive washing. Binding was expressed as the percentage of titer of virus bound to protein G cells to

FIG. 2. Demonstration of hCD55 incorporation on VSV particles by a viral pull-down assay. VSV harvested through HeLa, TgPAE A, and PAE E cells in the presence of antibody was incubated with protein G-expressing bacterial cells (OMNISORB). Antibodies used were three anti-human CD55 antibodies, BRIC 216, BRIC 471, and anti-DAF; anti-human CD46 J4-48 (hCD46); and anti-CD59 BRA-10G (hCD59). Five micrograms of anti-DAF and 1 µg of the other antibodies were used for incubation with virus and protein G cells. Titers of VSV for pulled-down and input particles were determined by TCID₅₀ assay. The ratio of these titers is shown as percent binding.

input titers (Fig. 2). TgPAE/VSV and HeLa/VSV but not PAE/VSV virus was pulled down with anti-hCD55 antibodies. Protein G cells with “irrelevant” anti-hCD46 and anti-hCD59 antibodies bound only HeLa/VSV, since these hCRPs are expressed on HeLa cells. These results indicated that infectious VSV particles harvested from TgPAE cells incorporate human CD55 molecules.

Complement sensitivity of MLV-A harvested from TgPAE and PAE cells. To test the complement sensitivity of MLV-A virus, a LacZ reporter assay was used. LacZ vectors packaged in MLV-A particles, harvested from TgPAE, PAE, and HeLa cells, were incubated in an equal volume of human serum but with various amounts of fresh or heat-inactivated human serum for 1 h at 37°C and then titrated on human TE671 cells. Virus infection, detected by β-galactosidase staining after treatment with 50% fresh serum or its twofold dilutions in heat-inactivated serum, was expressed as the percentage of titer relative to that after control treatment with 50% heat-inactivated serum and plotted against the final percentage of fresh serum in the virus-serum reaction mixture (Fig. 3A). While there was no significant reduction of the titer of MLV-A from HeLa cells across the dilution of fresh serum, viruses from both PAE and TgPAE were inactivated at high concentrations of fresh serum. This is consistent with our previous observation that α-Gal-free retrovirus particles, such as those released from the HeLa cells, are resistant to complement and that the presence of α-Gal is the dominant determinant of virus sensitivity to killing at high concentrations of fresh human serum (48, 49). This is further supported by our experiments using VSV harvested from IOWA Gal-null cells (see below).

Viruses from TgPAE and PAE cells showed different sensitivities to serum at lower complement concentrations. A higher proportion of MLV-A produced by all three independent TgPAE cell cultures expressing hCD55 than those from non-transgenic porcine cells (PAE) survived the same serum treat-

FIG. 3. MLV-A sensitivity to human complement. (A) LacZ(MLV-A) harvested from three transgenic (TgPAE A, B, and C) and three nontransgenic (PAE D, E, and F) primary pig cells was assayed for its sensitivities to human complement by an infection assay. Virus harvested from HeLa cells is inherently resistant to human complement and was used as a control in two different experiments. Mean percentage titers from duplicated experiments relative to those achieved when using heat-inactivated human serum are shown. (B) Three different human sera, serum 1 (straight line), serum 2 (dotted line), and serum 3 (dashed line), were tested for their ability to inactivate MLV-A harvested from TgPAE A or PAE D cells. Virus harvested from HeLa cells was used as a control. Titers are expressed as a mean percentage relative to titer achieved using heat-inactivated serum from duplicated experiments.

ment at the range of 25% fresh serum concentration and below (Fig. 3A). However, virus from TgPAE A and C was slightly more protected than that from TgPAE B, and this may be the effect of different levels of CD55 expression on cells from the three pigs. More complement activity was required to inactivate MLV-A from TgPAE (TgPAE/MLV-A) than from PAE (PAE/MLV-A), as shown by estimation of the fresh serum concentrations at which there is 90% inhibition of MLV-A infection (IS_{90}) in Table 1. This partial protection from complement-mediated inactivation of TgPAE/MLV-A compared to results with PAE/MLV-A is presumably due to the effect of human CD55 in virus harvested from the transgenic cells.

To establish whether complement-mediated inactivation varied between individuals, MLV-A packaging the LacZ vector was harvested from TgPAE A, PAE D, and HeLa cells and treated as described above with fresh serum samples from three different individuals (serum 1, 2, and 3). The percentage of titer relative to that of heat-inactivated serum was plotted against the final percentage of fresh serum in the virus-serum reaction mixture (Fig. 3B). All sera showed comparable activities.

FIG. 4. PERV sensitivity to human complement. PERV-A 14/220 harvested from two transgenic pig cell lines, TgPAE A and TgPAE B, and one nontransgenic pig cell line, PAE D, were tested for their sensitivities to human sera 1 and 3. Virus harvested from HeLa cells was used as a control for resistant virus. Titers are expressed as a mean percentage relative to titers achieved using heat-inactivated serum of duplicated experiments.

MLV-A produced from HeLa cells (HeLa/MLV-A) was resistant to complement-mediated inactivation in each of the three different sera at all serum concentrations, as expected (Fig. 3B). TgPAE/MLV-A and PAE/MLV-A both showed a reduction in titer; although infection varied with the different sera, the trend was similar (Fig. 3B). TgPAE/MLV-A was more protected in all three sera than PAE/MLV-A and retained 10% infectivity at a fresh serum concentration as high as 12%, while PAE virus did not retain 10% infectivity when the fresh serum concentration was above 2.8% (data not shown). Thus, TgPAE/MLV-A appeared to have a significant degree of protection from complement-mediated inactivation compared to PAE/MLV-A, and the trend of protection is common to different individual sera.

Complement sensitivity of PERV harvested from TgPAE and PAE cells. TgPAE (A and B), PAE (E), and HeLa cells were infected with a high-titer PERV 14/220 isolate (12, 26) by cocultivation. Virus was incubated for 1 h with both fresh (50% and twofold dilutions in heat-inactivated serum) and heat-inactivated (50%) human serum 1 or 3 and used to infect 293 target cells. HeLa/PERV, like HeLa/MLV-A, was unaffected by human complement and remained infectious at the highest fresh serum concentration of 50% (Fig. 4; Table 1). Both TgPAE/PERV and PAE/PERV were susceptible to complement-mediated inactivation (Fig. 4), although TgPAE/PERV appeared less sensitive; TgPAE/PERV retained infectivity at fresh serum concentrations as high as 20%, while less than 10% infectivity of PAE/PERV was retained at a fresh serum concentration as low as 5.0% (Table 1).

Complement sensitivity of VSV harvested from TgPAE and PAE cells. VSV was used to infect TgPAE (A and C), PAE (D and F), ST-IOWA (wild-type), ST-IOWA Gal-null, and HeLa cells. Virus was harvested from the cells in serum-free medium and incubated for 1 h at 37°C with an equal volume of both fresh (50% and twofold dilutions in heat-inactivated serum) and heat-inactivated (50%) human serum 1. Virus was then titrated by a TCID₅₀ assay. HeLa/VSV was resistant, while VSV harvested from PAE cells was susceptible to human complement-mediated inactivation, as expected (Fig. 5A). TgPAE/

A

B

FIG. 5. Sensitivity of VSV to human complement. (A) VSV harvested from two transgenic pig cells, TgPAE A and TgPAE C, and two nontransgenic pig cells, PAE D and PAE F, were tested for their sensitivities to human serum 1. Virus harvested from HeLa cells was used as a positive control. (B) VSV harvested from ST-IOWA Gal^{+/+} (IOWA-Gal+/VSV) or ST-IOWA Gal^{-/-} (IOWA-Gal-/VSV) cells was tested for sensitivity to human complement using serum 1. HeLa/VSV virus was used as a positive control. TCID₅₀ titers are expressed as a mean percentage relative to titers achieved using heat-inactivated serum from duplicated experiments.

VSV was less sensitive to complement-mediated inactivation and was infectious at higher fresh serum concentrations (IS₉₀, 16 to 38%) than PAE/VSV (IS₉₀, 5 to 10%) (Table 1).

Complement sensitivity of VSV harvested from pig cells with or without α -Gal epitope. To confirm that virus inactivation by human serum is mainly mediated by α -Gal epitope and anti- α -Gal natural antibodies, we employed the porcine testis cell line ST-IOWA and its derivative (ST-IOWA Gal-null) lacking the α -galactosyl transferase gene (*GGTA1*) (37). While Gal-null cells have been shown to produce PERV resistant to human serum (37), VSV passaged through these cells was tested in this study. ST-IOWA Gal-null cells produced VSV resistant to complement-mediated inactivation, although at the highest serum concentration (50%) there was some degree of inactivation, implying that although α -Gal is the major xeno-reactive epitope, there are other minor xeno-reactive epitopes (9). VSV from wild-type ST-IOWA was more sensitive, with approximately a 10-fold difference in sensitivity between Gal-null (Gal^{-/-}) and Gal-positive (Gal^{+/+}) ST-IOWA cells (Fig. 5B).

DISCUSSION

In this study, we address the effects of CD55 expression and α -Gal epitopes on the sensitivity of three viruses to human serum inactivation. The possibility of viruses budding either from these CD55-transgenic pig cells or from Gal-null pig cells, thereby attaining a degree of protection from the antiviral effects of human complement, was investigated.

The incorporation of host cell molecules into retrovirus envelopes is a well-known phenomenon. Human immunodeficiency virus and simian immunodeficiency virus budding from infected cells are known to incorporate cellular proteins, such as HLA or adhesion molecules, into their envelopes (27). Molecules from the host cell can be selectively incorporated into viral particles (52, 53), including glycosylphosphatidylinositol-anchored proteins (39, 42). Such proteins have been found to localize to plasma membrane lipid "rafts," which are glycolipid-enriched membrane domains organized on the cell surface enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol-linked proteins that serve as moving platforms on the cell surface (44). Viruses budding from primate cells can incorporate membrane-attached CRPs into their envelopes. Human cytomegalovirus, human immunodeficiency virus, simian immunodeficiency virus, and human T-lymphotropic virus type 1 are all known to incorporate human CRPs into their envelopes and consequently have shown resistance to human complement (24, 42, 43, 45). We provide direct evidence that VSV infectious particles from TgPAE cells bear human CD55 molecules (Fig. 2).

This study demonstrates that MLV-A, PERV, and VSV virions harvested from hCD55 transgenic TgPAE cells are significantly protected from complement-mediated inactivation, compared to viruses harvested from nontransgenic PAE primary porcine cells. All TgPAE viruses retained infectivity at higher serum concentrations than PAE viruses. The most likely explanation for this observation is that transgenic human hCD55 (hDAF) molecules were incorporated into viral envelopes, as shown for VSV, and partially protected the viruses from serum inactivation. In clinical xenotransplantation settings, viruses produced by hCD55-transgenic cells might therefore expected to be inactivated more slowly than those produced by nontransgenic cells.

The α -Gal epitope is the main target for human serum attack, as has been shown previously (38, 41, 49), and as we have documented using virus from Gal-null cells. However, some sensitivity of particles remains at high serum concentrations, and this is probably due to the presence of other minor epitopes and antibodies (8, 9). α -Gal expression appeared to be somewhat lower in PAE cells than in TgPAE cells used in this study (Table 1). Thus, it is unlikely that the reduced sensitivity of virus from the TgPAE cells is due to reduction of the complement activation epitope. However, minor variations in sensitivity may be due to the variability of α -Gal epitope expression in different pigs and subsequent incorporation of the epitope into the viral envelope.

In a recent study (46), the implications of using transgenic pigs for xenotransplantation was assessed by transfecting ST-IOWA cells with a human CD59 (hCD59) vector. PERV, exogenously infected, was shown to incorporate hCD59 into its envelope by a protein A immunocapture assay. While these

viruses were subsequently protected against human complement-mediated virolysis, as measured by reverse transcriptase release, hCD59 incorporation had no effect on the inactivation of PERV by human serum at a 1:10 human serum dilution (46). This is in contrast to our data using viruses from hCD55-transgenic cells, since we detected significant protection at different serum dilutions. It may be interesting to test viruses containing hCD59 at lower serum concentrations to examine the possibility that hCD59 also has a protective effect on virus infectivity. CD59 interferes with the final assembly of the membrane attack complex (MAC), and previous studies by our laboratory have shown that human sera deficient in complement components required for MAC formation, C7 and C9, were still able to inactivate gammaretroviruses produced from α -Gal-expressing host cells (47). Thus, CD59 may not prevent virus inactivation, although it inhibits viral envelope lysis. In contrast, hCRPs such as CD55 that act further upstream in the complement cascade accelerate the decay of C3 convertases (19), thereby interfering with the deposition of complement on virions. These viruses are at least partially protected from complement-mediated inactivation, as demonstrated in this study. The exact mechanism by which this viral infectivity is inactivated remains to be elucidated.

The protection of MLV-A, PERV, and VSV harvested from TgPAE cells to inactivation by human complement was apparent only after sera were diluted at least 1:8. Previous studies with other viruses have shown protection from complement-mediated virolysis at much higher serum concentrations (24, 42, 43, 45). In these studies, virus was harvested from human cells that express more than one hCRP, including CD55, CD59, and CD46. In our study, singly transgenic pigs cells were used; however, with the current advancement in biotechnology, the generation of pigs transgenic for more than one hCRP, in order to minimize HAR, is also possible. Virus budding from these pigs could be resistant to complement inactivation at higher serum concentrations due to the additive protective effect of the CRPs.

The development of pigs with a knockout of one allele of the α -galactosyl transferase gene, hence the α -Gal epitope, has now been done by nuclear transfer techniques (10, 17, 35). More recently, pigs with a knockout of both alleles of the α -galactosyl transferase gene have been generated (32). We examined pig cells in which expression of the α -Gal epitope was knocked out. VSV propagated through these cells was insensitive to complement-mediated inactivation. The accompanying paper (37) demonstrates reduced complement-mediated inactivation of PERV. However, there are still the minor epitopes, which could bind to trigger the complement cascade. The next step towards minimizing HAR in xenotransplantation may be the generation of homozygous α -Gal knockout, hCRP-transgenic pigs. Enveloped viruses budding from such pigs, whether endogenous (e.g., PERV) or exogenous, will probably have a significant degree of resistance to human complement-mediated inactivation, thereby raising the potential risk of zoonosis in xenotransplantation (6, 50).

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