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THE IMPACT OF SERUM INCUBATION TIME ON IgM/IgG BINDING TO PORCINE AORTIC ENDOTHELIAL CELLS

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Abstract

Background—The results of the assay for measuring anti-nonGal antibodies (which affect pig xenograft survival) in recipients is important. Serum incubation time and concentration may be important factors in the extent of antibody binding to the graft. The aim of this *in vitro* study was to determine the optimal incubation time and serum concentration for measuring anti-nonGal antibody binding to porcine aortic endothelial cells (pAECs).

Materials and Methods—Pooled human, naïve and sensitized baboon sera were incubated with wild-type, α 1,3-galactosyltransferase gene-knockout (GTKO), and GTKO/human CD55 pAECs. IgM/IgG binding to pAECs after varying serum incubation times (0.5, 1, 2, 3h) and concentrations (5, 10, 20, 40 μ L) was determined by flow cytometry.

Results—An increase in incubation time from 30min to 2h was associated with increases in anti-nonGal IgM/IgG binding to GTKO and GTKO/hCD55 pAECs of pooled human, naïve and sensitized baboon sera ($P < 0.05$). Pooled human serum showed a significant increase in anti-nonGal IgM ($\times 1.5$), and a minimal increase in anti-nonGal IgG antibody binding. IgM/IgG binding of sensitized baboon serum to GTKO pAECs after 2h incubation was $\times 1.5$ and $\times 2$ greater than after 30min incubation, respectively whereas naïve baboon sera showed minimal (non-significant) increase in anti-nonGal IgM/IgG antibody binding. With 2h incubation, increasing the serum concentration from 5 μ L to 20 μ L significantly increased antibody binding to nonGal antigens in pooled human and sensitized baboon serum. With naïve baboon serum, only IgG was significantly increased.

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Conflict of interest

The authors of this manuscript have no conflicts of interest to disclose.

Conclusions—Increasing the serum incubation time contributed to improve the sensitivity of detecting anti-nonGal antibodies, without affecting cell viability *in vitro*.

Keywords

α 1, 3-galactosyltransferase gene-knockout; Antibodies, anti-nonGal; Baboon; Human; Porcine aortic endothelial cells; Xenotransplantation

Introduction

Xenotransplantation using genetically-engineered pig organs is a possible solution to the organ shortage, but antibody-mediated rejection in pig-to-nonhuman primate models is one of the barriers which need to be overcome before clinical application^{1,2}. All humans have antibodies directed against galactose- α 1,3-galactose (Gal) expressed on the pig vascular endothelium^{3,4}. These natural antibodies cause hyperacute rejection in wild-type (WT) pig-to-primate organ xenotransplantation, but this is rare after the transplantation of an organ from an α 1,3-galactosyltransferase gene-knockout (GTKO) pig^{5–7}. If a graft recipient has high levels of preformed anti-nonGal antibodies prior to transplantation, this will affect graft survival^{8,9}, and therefore it could be important to select patients with low levels of antibodies¹⁰.

The accurate measurement of anti-nonGal antibodies in recipients is therefore important, especially the extent of their binding to porcine aortic endothelial cells (pAECs) because pAECs are the main target cells following pig organ xenotransplantation¹¹. The binding of anti-pig antibodies to GTKO pAECs is less than to WT pAECs^{3,4,7}. An assay that differentiates low from high binding is very likely to be important when clinical trials of xenotransplantation are initiated. There have been several studies in which anti-nonGal antibody levels were measured, but there was considerable variation in methodology (e.g., different target cells, varying serum and cell concentrations, different incubation times) (Table 1).

Serum concentration is one important factor in measuring antibody levels, especially for anti-nonGal antibodies^{11,12}. However, there is no information on the relationship between serum incubation time and the binding of anti-nonGal antibodies. An incubation time of 30min has been used by many research groups, including our own (Table 1). The aim of the present study was to investigate whether incubation time and/or serum concentration plays an important role in increasing our ability to distinguish between low and high antibody binding to nonGal antigens.

Materials and Methods

Serum samples

All studies using human serum were approved by the Research Ethics Committee of the University of Pittsburgh. Sera were collected from 10 healthy human volunteers of all ABO blood types who had no history suggesting previous exposure to pig antigens (except in the diet) or to alloantigens (i.e., no previous pregnancies, blood transfusions, or organ allotransplants). Serum from one sensitized baboon (B64–14) was used as a positive control.

This baboon had previously received an aortic patch transplant from a GTKO pig without immunosuppressive therapy, as previously described¹³. Sera from 5 naïve baboons were collected (Division of Animal Resources, Oklahoma University Health Sciences Center, Oklahoma City, OK, USA). Decomplementation of serum was carried out by heat-inactivation for 30min at 56°C, and sera were stored at -80°C until use.

Cell sources and preparation of pig aortic endothelial cells (pAECs) and human aortic endothelial cells (hAECs)

pAECs were collected from WT pigs and from two different genetically-modified pigs provided by the National Swine Resource and Research Center (NSRRC, Columbia, MO), namely GTKO and GTKO/hCD55 pigs. pAECs were isolated from fresh aortas and cultured, as previously described¹⁴. Human aortic endothelial cells (hAECs) were purchased from Lonza (Walkersville, MD), and cultured as previously described¹⁴.

Expression of Gal and hCD55 on pAECs and hAECs

Surface expression of Gal and hCD55 was determined by flow cytometry, as previously described³.

Detection of serum IgM/IgG binding to pAECs and hAECs

IgM and IgG binding to WT, GTKO, and GTKO/hCD55 pAECs and hAECs was determined by flow cytometry, as previously described with some modification³. Briefly, pAECs or hAECs were resuspended in staining buffer (PBS containing 1% bovine serum albumin [Invitrogen, Carlsbad, CA] and 0.1% NaN₃) to a final cell concentration of 1×10^6 cells/mL. pAECs and hAECs (0.1×10^6 cells/100µL) were then transferred into each tube and incubated with different volumes (40, 20, 10, 5µL) of heat-inactivated human or sensitized baboon serum, diluted in staining buffer to 28.6%, 16.7%, 9%, 4.8% serum final concentration (for hAECs as a negative control, a single serum concentration of 20µL was used), for different periods of incubation (0.5, 1, 2, or 3h) at 4°C. After incubation, the cells were washed twice with staining buffer, and centrifuged at 600g at 4°C for 5min. Cells were then resuspended in 10µL of heat-inactivated goat serum in 100µL staining buffer for 20min at 4°C, to prevent nonspecific binding, and incubated further with 10µL fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM (µ chain-specific) and IgG (γ chain-specific) at 1:50 dilution (Invitrogen) for 30min in the dark at 4°C. Finally, the cells were washed with staining buffer, centrifuged at 600g at 4°C for 5min, and then added to 200µL staining buffer.

Dead cells were stained with 10µL (1:25 concentration) propidium iodide (BD Biosciences, San Jose, CA) prior to flow cytometry. Antibodies binding to live cells were measured by LSR II flow cytometry (BD), and analyzed by FlowJo software (Treestar, Ashland, OR). Negative controls were obtained by incubating the target cells with secondary anti-human antibodies only (with no plasma). Binding of IgM and IgG was measured using the relative geometric mean, which was calculated by dividing the geometric mean value for each sample by the negative control, as previously described¹⁵.

Statistical methods

Values are presented as mean $\pm$ SEM. The statistical significance of differences was determined by Student's t-test or nonparametric tests, as appropriate. The statistical tests were carried out using GraphPad Prism version 6 (GraphPad software, San Diego, CA). Differences were considered to be significant at $P<0.05$.

Results

Expression of Gal and hCD55 on WT, GTKO, GTKO/hCD55 pAECs and hAECs

WT pAECs expressed Gal, but GTKO and GTKO/hCD55 pAECs and hAECs were negative for Gal expression (Figure 1). GTKO/hCD55 and hAECs were positive for hCD55 expression. All WT, GTKO, GTKO/hCD55 pAECs and hAECs were positive for CD31 (vascular endothelial cell) expression ($>90\%$).

Effect of serum incubation and concentration on IgM/IgG antibody binding to WT pAECs

Antibody binding to WT pAECs was measured using (i) pooled human serum and (ii) sensitized baboon serum at two different serum concentrations - 16.7% (20 μ L) and 4.8% (5 μ L) with different serum incubation times (0.5, 1, 2, 3h) (Figure 2). Increasing the duration of serum incubation and concentration of both pooled human serum and sensitized baboon serum increased IgM antibody binding to WT pAECs, but made little difference to IgG binding (Figure 2). In pooled human and sensitized baboon serum, there was a significant difference in IgM binding between 20 μ L and 5 μ L serum concentration ($P<0.05$), but no significant difference in IgG binding. When incubation was increased from 30min to 2h (at a serum concentration of 20 μ L), there was increased IgM, but not IgG, binding to pAECs in pooled human serum. Increasing the incubation time to 3h did not increase IgM or IgG binding further. In contrast, sensitized baboon serum showed no significant difference in both IgM and IgG binding with incubation time although there was a trend of increased IgM and IgG binding to WT pAECs after 2h incubation.

Comparison of serum IgM/IgG antibody binding to WT, GTKO, and GTKO/hCD55 pAECs

The difference between GTKO and GTKO/hCD55 pAECs is only the expression of human CD55. We measured pooled human and sensitized baboon antibody binding to WT, GTKO, and GTKO/hCD55 pAECs. At 16.7% (20 μ L) serum concentration and 2h incubation, IgM binding to WT pAECs was significantly greater than to GTKO pAECs ($P<0.05$), but not IgG (Figure 3). There was no significant difference in antibody binding between GTKO and GTKO/hCD55 pAECs.

Effect of serum incubation time and concentration on IgM/IgG antibody binding to GTKO pAECs

Comparison of IgM and IgG antibody binding to GTKO (or GTKO/hCD55) pAECs with different serum concentrations (20, 5 μ L) and incubation times (0.5, 1, 2, 3h) was determined (Figure 4). Increasing the duration of incubation (up to 2h) and concentration (from 5 to 20 μ L) was associated with a significant increase in IgM and IgG binding to GTKO (and GTKO/hCD55) pAECs ($P<0.05$). There was no significant difference in IgM or IgG

antibody binding when incubation was extended from 2 to 3h. The greatest IgM binding to GTKO pAECs was at 20 μ L serum concentration and 2h incubation. The greatest IgG binding was at 20 μ L and 3h.

As a negative control, 20 μ L pooled human serum was incubated with hAECs for 0.5, 1, 2, or 3h. There was no increased nonspecific IgM or IgG antibody binding to hAECs after longer incubation times (data not shown).

Effect of serum concentration on IgM/IgG antibody binding to GTKO pAECs

Comparison of serum concentration on antibody binding to GTKO pAECs for 30min or 2h incubation was performed (Figure 5). Diluted sera (40, 20, 10, 5 μ L, i.e., 28.6%, 16.7%, 9%, 4.8%) were used to measure IgM and IgG binding to pAECs from GTKO, and GTKO/hCD55 pigs (n=7) following incubation for 30min or 2h.

After incubation of pooled human serum for 2h, IgM binding to GTKO pAECs of 20 μ L serum concentration was increased compared with 5 μ L and 10 μ L ($P<0.05$). Increasing the serum concentration did not affect IgG binding. Increasing the pooled human serum concentration to 40 μ L decreased both IgM and IgG binding ($P<0.05$).

After 2h incubation, IgM and IgG binding of sensitized baboon serum was not affected statistically by an increase in concentration, but a concentration of 20 μ L was associated with the highest IgM and IgG binding. An incubation time of 2h and a serum concentration of 20 μ L was the optimal choice for maximal antibody binding to GTKO and GTKO/hCD55 pAECs.

Effect of serum incubation time and concentration on naïve baboon serum IgM/IgG antibody binding to GTKO/hCD55 pAECs

Naïve baboon serum IgM and IgG antibody binding to GTKO/hCD55 pAECs at two serum concentrations (5 μ L, 20 μ L) and two incubation times (0.5h, 2h) was carried out (Figure 6). Prolonging the incubation time from 0.5h to 2h was associated with significant increases in IgM and IgG binding to pAECs at both serum concentrations (5 μ L, IgM $P<0.01$, IgG $P<0.05$; and 20 μ L, IgM $P<0.05$, IgG $P<0.01$). There was no significant difference in IgM and IgG binding to pAECs between 5 μ L and 20 μ L serum concentrations at different incubation times, except that IgG binding to pAECs increased at an incubation time of 2h ($P<0.05$).

These results indicated that increasing the duration of serum incubation, but not always of serum concentration, was associated with a significant increase in naïve baboon IgM and IgG binding to GTKO/hCD55 pAECs.

Effect of incubation time

Cell viability/cell death in pAECs—To determine the effect of incubation time and serum concentration on cell viability, the number of dead pAECs was measured by propidium iodide staining (n=12). There was no significant difference in GTKO pAEC viability with incubation time or serum concentration (Figure 7) indicating that a greater serum concentration or longer incubation time did not induce cell death. (Similarly, a longer

incubation time or an increase in serum concentration did not affect WT pAEC viability [data not shown].)

Discussion

Both preformed and elicited/induced antibodies which react to nonGal antigens on GTKO pAECs contribute to xenograft rejection^{2,7}. Therefore, it is important to investigate the presence of preformed natural antibodies against nonGal pig antigens in recipients before transplantation, and monitor their de novo induction after xenotransplantation. The levels of anti-nonGal antibodies, especially preformed natural antibodies, are significantly lower than those to WT pAECs, which express both Gal and nonGal antigens^{3,14}. It might be difficult to detect de novo induced anti-nonGal antibodies in recipients receiving immunosuppressive drug therapy^{5,9}. Therefore, it may be important to modify the assay to more optimally detect anti-nonGal antibodies *in vitro*.

There has been considerable variation in the *in vitro* assays aimed at measuring antibody binding to pig cells (Table1). Serum incubation for 30min has been the most common time used^{3,10–12,14–37}. A uniform method to measure natural and induced antibody responses to nonGal epitopes was recommended in 2014¹¹. In this study, differences in serum concentration affected antibody binding to nonGal antigens, which we have confirmed in our present study. However, the serum incubation time was not investigated as a variable. In our study, we found that serum incubation time also influenced antibody binding to non-Gal antigens.

The present study confirmed that antibody binding to GTKO and GTKO/hCD55 pAECs is significantly less than to WT pAECs, which supports our previous observations³. Increasing the serum concentration from 5 to 20 μ L was associated with increased IgM antibody binding to pAECs, but increasing the concentration further to 40 μ L was not advantageous in both pooled human and sensitized baboon serum. Furthermore, increasing the duration of incubation to 2h was associated with an increase in IgM and IgG antibody binding to GTKO pAECs, but a further increase to 3h did not increase binding. A serum concentration of 20 μ L (16.7%) and an incubation time of 2h proved optimal for IgM and IgG antibody binding to GTKO pAECs in both pooled human and sensitized baboon serum. Furthermore, we demonstrated that a longer serum incubation time and a higher concentration were not associated with toxicity to the GTKO cells either when we used pooled human serum or sensitized baboon serum.

The optimal serum incubation time and concentration for detecting anti-nonGal antibodies may differ under different assay conditions (e.g., type of serum sample [naïve or highly-sensitized]). In addition to pooled human and sensitized baboon serum, naïve baboon sera were tested at different incubation times and concentrations. Although the levels of anti-nonGal antibodies in these naïve baboons were 2–3 times lower for IgM and 10–15 times lower for IgG compared to those in sensitized baboon serum, a longer incubation time was associated with a significant increase in both IgM and IgG antibody binding to GTKO pAECs. These results confirmed that a longer incubation time increased anti-nonGal antibody binding to GTKO pAECs.

In contrast to the serum incubation time, when naïve baboon sera were tested, there was no significant difference in anti-nonGal IgM (for both 0.5h and 2h) or IgG (for 0.5h) antibody binding to GTKO pAECs between serum concentrations of 5 μ L and 20 μ L, although there was higher IgG binding to GTKO pAECs when 20 μ L sera were used.

These results suggest that the optimal concentration of serum samples for an antibody-binding assay might differ with different samples (e.g., naïve vs highly-sensitized). We cannot fully explain why an increase in incubation time led to an increase in anti-nonGal antibody binding. It may just be associated with a longer period to allow antibody to bind, but it is possible that the affinity of antibody to nonGal antigens is lower compared with that to Gal antigens, and therefore it takes longer for binding to occur to nonGal antigens.

It was important to exclude whether increased antibody binding was related to an increase in nonspecific binding. There was no increase in nonspecific antibody binding to human AECs, indicating that any increase in antibody binding to GTKO pAECs was specific to nonGal antigens on pAECs. Incubation time also proved to be a critical factor in antibody binding to nonGal antigens. For example, 20 or 40 μ L of serum incubated for 30min showed comparable binding to 5 and 10 μ L of serum incubated for 2h (Figure 5).

In summary, after deletion of Gal antigens through genetic engineering, nonGal antigens are still bound by primate antibodies. Since a longer serum incubation time (2h) is associated with an increase in antibody binding of pooled human, naïve and sensitized baboon sera without affecting cell viability, 2h incubation might prove beneficial in detecting anti-nonGal antibodies in naïve or sensitized recipients pre- and/or post-transplantation. This will be important when clinical trials of xenotransplantation are initiated, when the first patients may be selected on the basis of low anti-pig antibody levels. The previous published consensus protocol (11) eliminated much variability in the results obtained in different laboratories, but the current study indicates that the results of the assay might be improved by prolonging the incubation time.

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Abbreviations

GTKO	α 1,3-galactosyltransferase gene-knockout
GTKO/hCD55	α 1,3-galactosyltransferase gene-knockout pigs transgenic for the human complement-regulatory protein, CD55
pAECs	porcine aortic endothelial cells
WT	wild-type

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Figure 1.

Expression of Gal, hCD55 and CD31 on WT, GTKO, and GTKO/hCD55 pAECs and hAECs.

Cultured pAECs and hAECs were stained with BSI-B4 (for Gal), anti-human CD55 (clone IA10, BD), and rat anti-mouse CD31 (clone TLD-3A12, BD). The expression of Gal, hCD55 and CD31 on AECs was measured by flow cytometry.

Figure 2.

Pooled human and sensitized baboon serum IgM/IgG binding to WT pAECs (n=3, repeated ×3). Antibody binding to pAECs was measured using pooled human and sensitized baboon serum at two concentrations - 16.7% (20μL) and 4.8% (5μL) – and with different serum incubation times (0.5, 1, 2, or 3h). *indicates the result at each time-point compared with 0.5h (*P<0.05). # indicates the result of 5μl compared with 20μl at all time-points (#P<0.05; ##P<0.01).

Figure 3.

Comparison of serum IgM and IgG binding to WT, GTKO, and GTKO/hCD55 pAECs at 16.7% (20 μ L) serum concentration and 2h incubation (n=3, repeated $\times$ 3). (*P<0.05; n.s.= not significant)

Figure 4.

Pooled human and sensitized baboon serum IgM/IgG binding to GTKO or GTKO/hCD55 pAECs (n=12, repeated ×6). Antibody binding to pAECs was measured using pooled human and sensitized baboon serum at two concentrations - 16.7% (20µL) and 4.8% (5µL) – and with different serum incubation times (0.5, 1, 2, or 3h). *indicates the result at each time-point compared with 0.5h (*P<0.05; **P<0.01). # indicates the result of 5µl compared with 20µl at all time-points (#P<0.05; ##P<0.01).

Figure 5.

Pooled human and sensitized baboon serum IgM/IgG binding to GTKO or GTKO/hCD55 pAECs (n=7: repeated ×4). Antibody binding to pAECs was measured using pooled human and sensitized baboon serum at four concentrations - 28.6% (40μL), 16.7% (20μL), 9% (10μL), 4.8% (5μL) – and with two different serum incubation times (0.5 or 2h). (*P<0.05; n.s.= not significant)

Figure 6.

Naïve baboon sera IgM/IgG binding to GTKO/hCD55 pAECs. Antibody binding to pAECs was measured using naïve baboon sera (n=5) at two concentrations - 16.7% (20µL) or 4.8% (5µL) – and at two incubation times (0.5h or 2h). (*P<0.05; **P<0.01; n.s.= not significant)

Figure 7.

Cell viability/cell death of GTKO and GTKO/hCD55 pAECs after exposure to pooled human serum or sensitized baboon serum at two different incubation times and at four different serum concentrations (n=12: repeated $\times 6$). (n.s.= not significant)

Table 1

Pig cell types and human serum concentrations and incubation times employed by selected research groups in *in vitro* assays to measure human IgM/IgG antibody binding to pig cells (2006–2016).

Group	Target Cells	Concentrations	Incubation Times (hours)	Authors/Year
1	RMECs (WT, GTKO/, GTKO/NeuGcKO)	25%, 6.3%, 1.6%	1	Wang 2016 ³⁵
	PBMCs (GTKO, and GTKO/iGb3sKO)	25%	1	Butler 2016 ¹⁷
	PBMCs (GTKO/NeuGcKO, GTKO/NeuGcKO/hCD55, and GTKO/NeuGcKO/B4GalKO)	25%	1	Butler 2016 ¹⁶
	RBC (GTKO, GTKO/NeuGcKO and GTKO/NeuGcKO/β4GalKO)	25%	0.5	Wang 2016 ³⁶
	PBMC (GTKO, GTKO/NeuGcKO and GTKO/NeuGcKO/β4GalKO)	25%	0.5	Estrada 2015 ²¹
	RBC (GTKO, GTKO/NeuGcKO)	25%, 6.3%, 1.6%, 0.4%	1	Wang 2014 ¹²
	PBMCs (GTKO, GTKO/NeuGcKO)	25%	/	Burlak 2014 ¹⁰
	PBMC (GTKO/, GTKO/NeuGcKO)	25%	2	Lutz 2013 ²⁹
	pAEC (GTKO)	0–50% diluted	1.5	Chihara 2013 ²⁰
2	RBC, PBMCs, pAECs, CECs (WT, GTKO/CD46, and GTKO/NeuGcKO/hCD46)	20%	0.5	Lee 2016 ¹⁴
	CECs, pAECs (WT, GTKO, GTKO/NeuGcKO)	20% for IgM, 5% for IgG	1	Lee 2015 ¹⁵
	pAECs (GTKO)	20%	0.5	Ezzelarab 2012 ²³
	RBC (WT, GTKO)	5%	0.5	Long 2009 ²⁸
	pAECs, PBMC (WT, GTKO, GTKO/hCD46, hCD46)	20% or serial diluted	0.5	Hara 2008 ³
	PBMCs (WT GTKO)	20%	0.5	Rood 2007 ³²
	PBMC (WT, GTKO)	20%	0.5	Hara 2006 ²⁴
	PBMCs (WT, GTKO)	20%	0.5	Rood 2006 ³¹
	PBMC (WT GTKO)	20%	0.5	Ezzelarab 2006 ²²
3	pEC69 (GTKO)	10%	0.5	Kang 2015 ²⁵
4	pAECs (WT/GTKO)	25%, 6.3%, 1.6%, 0.4%	0.75	Azimzadeh 2014 ¹¹
5	pAECs (GTKO)	20%–2.5%	0.75	Bryne 2014 ¹⁸
	pAECs (GTKO)	50μl	0.75	Byrne 2008 ¹⁹
6	pECs (WT)	20%	1	Zhu 2008 ³⁷
7	pAEC (WT, GTKO)	25% for GTKO, 3% for WT	0.5	Scobie 2013 ³³
8	PBMC (GTKO)	10%	0.5	Liang 2013 ²⁷
	PBMC, PAEC (GTKO)	9%	0.5	Wong 2006 ³⁸
9	pAECs (hCD55, hTM, hCD55/hTM, hCD55/GTKO, and hCD55/hTM/GTKO)	50%	0.5	Miwa 2010 ³⁰
10	LSECs (GTKO, WT)	50%, 5%, 0.5%	0.5	van Poll 2010 ³⁴

* Table compiled from data from 28 publications from 10 different research groups

LSECs = Liver Sinusoidal Endothelial Cells

pEC = porcine Endothelial Cells

pAECs = pig Aortic Endothelial Cells

pCEC = pig Corneal Endothelial Cells

pEC69 = pig Endothelial Cell Lines 69

RMECs= Renal Microvascular Endothelial Cells

RBCs = Red Blood Cells Peripheral Blood Mononuclear Cells