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ANTI-GAL ANTIBODIES IN α 1,3-GALACTOSYLTRANSFERASE GENE-KNOCKOUT PIGS

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INTRODUCTION

Most mammalian species (including New World monkeys, pigs, and mice) express galactose- α 1,3-galactose (Gal) abundantly on the surfaces of many cells.^{1–3} Gal expression results from the activity of an enzyme encoded by the α 1,3-galactosyltransferase gene (GGTA1).^{4–6} Certain mammalian species, such as catarrhines (humans, apes, and Old World monkeys), do not have a functional GGTA1 gene and correspondingly do not express Gal.^{7,8} The absence of Gal from catarrhines may have been due to a selective evolutionary event prompted by an infectious agent that occurred 28 million years ago.⁹ The function of Gal is unknown, but is clearly not essential for survival.

Gal is the major antigen expressed on pig cells to which primate natural (preformed) anti-pig antibodies (Abs) bind. This activates the complement system and results in hyperacute rejection in pig-to-primate transplantation models.^{3,10–12} Anti-Gal Abs are absent in mammalian species that express Gal. The development of natural Abs to Gal in species in which this antigen is not expressed has been attributed to a response to gastrointestinal bacteria/viruses that express Gal.¹³ In humans and baboons, anti-Gal Abs are not present at birth (except maternal-derived IgG), but develop during the first few months of life^{14–17}. IgM, IgG, and IgA anti-Gal Ab isotypes have been identified.^{3,12,18} In humans, anti-Gal Abs represent a major component of total immunoglobulin, with perhaps 1–5% of circulating immunoglobulins being directed to Gal.^{3,19,20}

Genetically-engineered mice and pigs in which the GGTA1 gene has been knocked out^{21–24}, and therefore which lack Gal, produce Abs to Gal.^{21,23,25,26} The production of anti-Gal Abs can be used as indirect confirmation of the successful deletion of Gal. Revivacor, Inc (Blacksburg, VA) has utilized somatic cell nuclear transfer in combination with gene targeting techniques to establish a genetically-engineered pig line that does not express Gal, i.e., α 1,3-galactosyltransferase gene-knockout (GTKO) pigs. This was accomplished by disruption of the pig GGTA1 locus mediated by a pPL657 vector that targeted exon 9, the location encoding the catalytic domain of the GGTA1 gene.²⁷ Homozygous inactivation of both alleles of GGTA1 results in an inactive enzyme.²³ This technology and subsequent breeding have resulted in a line of pigs intended as a source of material for use in clinical biomedical applications as well as organs and cells for clinical transplantation.

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The aim of the present study was to determine the production of anti-Gal IgM and IgG in the GTKO pig line, and to document whether any differences in Ab levels occurred with age or gender.

MATERIALS AND METHODS

Source Animals

Forty-seven GTKO pigs (30F, 17M) (Revivicor, Blacksburg, VA) that ranged in age from 10–801d were evaluated for genotype by long-range polymerase chain reaction (LR-PCR) on blood²⁷, and for the presence of serum Abs to Gal by ELISA (Table 1). Nineteen pigs were tested more than once, with at least several weeks between each collection of blood. Age, gender, and generation were known from breeding records.

Blood and sera were also obtained on 7 occasions from 4 healthy Large White/Landrace cross-breed wild-type (WT) adult female pigs of 210–420d old (the breed from which the GTKO pigs were derived). Three of the WT pigs were evaluated twice several weeks apart. Both sets of pigs were managed under normal husbandry conditions that adhered to standard guidelines for agricultural animals.²⁸

Sera

Blood was collected from the pigs through the jugular vein or anterior vena cava, and drawn into either 10ml vials containing K₂EDTA for genotype determination or into serum tubes without anticoagulant for Ab measurement. Serum was separated by centrifugation and placed in a vial. Samples showing significant hemolysis were excluded. Blood was stored at approximately 4°C, but genotyping was performed within approximately 24h (at Revivicor). Sera from both WT and GTKO pigs were frozen (-20°C) and, once a sufficient number of samples had accumulated, were sent overnight to the Starzl Transplantation Institute for measurement of anti-Gal IgM and IgG levels.

Measurement of Anti-Gal IgM and IgG Levels by ELISA

The serum specimens were thawed to room temperature. Levels of anti-Gal IgM and anti-Gal IgG were measured by ELISA, as previously described.^{29,30}

Measurement of Total IgM and IgG Levels by ELISA

Ninety-six well plates were coated with goat anti-pig IgM and IgG (Bethyl, Montgomery, TX) with a concentration of 0.5µg per well. The plates were incubated at room temperature for 1h. After washing, 3% BSA (Sigma, St. Louis, MO) blocking buffer was added and then incubated at room temperature for 1–1.5h. Serum samples were diluted to concentrations of 1:1000, 1:5000, 1:25,000 and 1:125,000 for IgM testing, and 1:25,000, 1:125,000, 1:625,000 and 1:3,125,000 for IgG testing. (Optimal dilutions were established by initially testing dilutions of 1:200, 1:1,000, 1:5,000, 1:25,000, 1:125,000, 1:625,000, 1:3,125,000, and 1:15,625,000 for both IgM and IgG.) Dilutions were added at 100µl/well in triplicate and then incubated at room temperature for 1h. Corresponding goat anti-Pig IgG and IgM HRP conjugated antibodies (Bethyl, Montgomery, TX) were added and then incubated at room temperature for 1h. After washing, 50µl of 3,3',5,5'-Tetramethylbenzidine (Sigma, St. Louis, MO) substrate were added to each well for color development, which was stopped after 6min by adding 50µl of sulfuric acid. The plates were then read at 450nm for 1sec per well to colorimetrically measure the absorbance of each well with a 1420 Multilabel Counter Victor³ (PerkinElmer, Waltham, MA). OD values of PBS were considered background, and were subtracted from the OD value of the samples.

Statistical Analyses

The effect of genotype (WT vs GTKO) and gender (GTKO male vs female) was evaluated by single factor analysis of variance (ANOVA) (Microsoft Excel, Redmond, WA). A difference of $p < 0.05$ was considered significant.

RESULTS

Genotyping

Genotyping confirmed the presence of the targeting construct mediated by vector pPL657 on both alleles of GGTA1 on all of the GTKO pigs. The targeting construct was confirmed absent on both alleles of GGTA1 for all 4 of the WT pigs, whereas all of the GTKO pigs possessed the targeting construct on both alleles of GGTA1.

Anti-Gal IgM and IgG Levels

Seven sera from the 4 WT pigs were used to establish baseline OD values (IgM 0.27; IgG 0.06) and represented the absence of Abs to Gal (Table 1 and Figure 1). Seventy-eight (78) serum samples derived from 47 GTKO pigs were evaluated (Table 1 and Figure 1). Significant differences in Ab levels for both IgM ($p < 0.05$) and IgG ($p < 0.05$) were observed between WT and GTKO pigs.

GTKO pigs were noted to have varying levels of anti-Gal Abs depending on the age of the pig (Figure 2). The timing of the peak levels of anti-Gal IgM and IgG was similar (at approximately 100 days), though there was much greater variation in IgG. Anti-Gal IgM Ab was detected in newborn GTKO piglets, and peaked at approximately 3–6m of age to more than double the level in the newborn; this higher level was maintained through to the oldest pigs evaluated ($>2y$) (Figure 2A).

Anti-Gal IgG Ab was also detected in newborn piglets, and increased as the pigs aged, peaking at approximately 3m to a level double that of the newborns (Figure 2B). In contrast to IgM, the IgG levels decreased slowly but steadily until they were almost undetectable at approximately 20m of age. There was a slight increase in the oldest pigs tested ($>2y$), which cannot be explained (although the number of sera tested was small).

At least 3 sequential measurements were made in a subgroup of 8 GTKO pigs that were followed over several months. The same patterns of IgM and IgG were seen as described above. No significant differences ($p > 0.05$) were detected in Ab levels for either IgM or IgG between GTKO males and females (not shown); the trends associated with age were seen in both sexes.

Total IgM and IgG Levels

In view of the reduction in anti-Gal IgG seen after 6m, we measured total IgM and IgG to determine whether the anti-Gal IgG pattern was common for all GTKO pig IgG. Thirty nine sera were selected to represent all ages of the GTKO pigs. Although total IgM followed the pattern seen with anti-Gal IgM (Figure 3A), total IgG did not decline after 6m, but remained stable in the pigs at later ages (Figure 3B). A further observation was that total IgG levels were relatively higher during the first few days of neonatal life than anti-Gal IgG levels.

DISCUSSION

GTKO pigs produce natural Abs to Gal, consistent with other species that do not express Gal antigens, e.g., humans, chimpanzees, and baboons.^{1,9,31} Identification of the presence of anti-Gal IgM and IgG Abs in GTKO pigs provides an indirect measure of the complete

absence of Gal in these animals. The level of the isotype varies depending on the age of the animal. The trends associated with age were consistent and durable between both males and females, and with repeated evaluation of single animals. The data provide useful baseline data for future experimental studies in GTKO pigs, e.g., relating to the Ab response to WT pig allografts.²⁶

GTKO pig natural Abs would appear to be similar to those in other species. They developed during the same period of infancy as those in humans and baboons¹⁷, and were shown to be cytotoxic (not shown).

There have been no previous studies in which anti-Gal Ab levels have been studied in such a large number of GTKO pigs at such frequent intervals for such prolonged periods of time. However, Griesemer et al.,²⁶ measured anti-Gal IgM and IgG in two GTKO miniature swine at 2, 5, 8, and 20m. A low level of IgM was present at 2m, with the titers increasing at 5m, and remaining high. IgG was not detectable until 5m, increased rapidly thereafter, and interestingly remained high in the single pig in which it was measured at 20m (in some contrast to the findings in the present study in which the mean level of IgG decreased after the first 6m).

In the study reported here, anti-Gal IgG and total igG were detectable 10 days after birth. As it has been reported that no maternal cells or immunoglobulin cross the six-layered placental barrier in the pig³², IgG was presumably introduced to the newborn through the colostrum of the sow. In studies of antigen uptake by ovalbumin-fed pigs, antigen feeding to the sow during lactation induced IgG Ab production in the offspring.³² In a previous study from our center, anti-Gal IgM was not detectable at 1 week of age in human neonates or at 6 weeks in infant baboons, but measurements at earlier time-intervals were not made.¹⁷ Anti-Gal IgG was present in the human neonates, but not in the infant baboons at these times. In subsequent studies in infant baboons, anti-Gal IgG has been detected in some baboons within the first month of life (Dons E, et al., unpublished data).

Whereas anti-Gal IgM and total IgM followed a similar pattern throughout the life of the GTKO pig, the level of anti-Gal IgG declined after 6m, in contrast to total IgG which remained stable. The reason for this difference remains unknown.

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Figure 1.

Mean $\pm$ SEM of anti-Gal antibody levels (IgM and IgG) for WT and GTKO pigs. The WT values represent the absence of antibodies to Gal. Significant differences were detected between the two different genotypes for both IgM ($p < 0.05$) and IgG ($p < 0.05$).

A: **B:** **Figure 2.**

(A): Variation in anti-Gal IgM levels as a function of animal age. Anti-Gal IgM antibodies were detected in newborn piglets (at 10 days of age – the earliest time measured), increased with age, peaking at approximately 4m of age to a level approximately double that of newborns. This higher level was maintained through to the oldest pigs sampled (>2y). The baseline level measured in WT pigs (indicating an absence of anti-Gal IgM) is indicated (dotted line).

(B): Variation in anti-Gal IgG levels as a function of animal age. Anti-Gal IgG antibodies were detected in newborn piglets (at 10 days of age – the earliest time measured), increased

with age; peaking at approximately 3m of age to a level approximately double that of newborns. In contrast to IgM, the IgG levels decreased and were almost undetectable in pigs of approximately 600 days of age. The baseline level measured in WT pigs (indicating an absence of anti-Gal IgG) is indicated (dotted line).

Figure 3.

(A): Variation in total IgM levels as a function of animal age. IgM was detected in newborn piglets (at 10 days of age – the earliest time measured), increased with age, peaking at approximately 9m of age to a level 2–3 times that of newborns. This high level was maintained through to the oldest pigs sampled (>2y).

(B): Variation in total IgG levels as a function of animal age. The total IgG level was high in newborn piglets (at 10 days of age – the earliest time measured), fell during the first month and then increased with age; peaking at approximately 9m of age to a level approximately

double that of that at 1m. This high level was maintained through to the oldest pigs sampled (>2y). Note the difference in scale between (A) IgM and (B) IgG levels

TABLE 1

Anti-Gal IgM and IgG Antibody Values in GTKO Pigs of Varying Ages and in Adult WT Pigs

Age (days)		IgM*	IgG*
Mean	SD	Mean(SE)	Mean(SE)
GTKO			
11 (n=13)	1.0	0.33 (0.08)	0.60 (0.12)
37 (n=6)	4.0	0.30 (0.13)	0.68 (0.32)
66 (n=12)	1.7	0.86 (0.08)	0.92 (0.13)
86 (n=9)	3.2	1.10 (0.07)	1.10 (0.17)
116 (n=11)	1.8	1.14 (0.09)	1.09 (0.16)
194 (n=6)	25.0	1.26 (0.04)	1.00 (0.36)
298 (n=10)	59.7	1.17 (0.06)	0.4 (0.11)4
626 (n=7)	19.4	1.21 (0.09)	0.17 (0.05)
795 (n=4)	5.6	0.94 (0.08)	0.66 (0.15)
191 (n=78)	223.4	0.90 (0.05)	0.76 (0.06)
WT			
305 (n=7)	59.1	0.27 (0.04)	0.06 (0.02)

*
(OD_{480nm})