Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Late onset of development of natural anti-nonGal antibodies in infant humans and baboons: implications for xenotransplantation in infants

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Keywords
α1,3-galactosyltransferase gene-knockout, anti-pig antibodies, infants, primates, xenotransplantation.

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Received: 17 May 2007
Revision requested: 12 June 2007
Accepted: 28 July 2007

doi:10.1111/j.1432-2277.2007.00546.x

Summary

If an ABO-incompatible heart is transplanted into an infant before natural antibodies have developed to the specific donor carbohydrate A/B antigen(s), then B-cell tolerance to the donor A/B antigen is achieved, and these antibodies never develop. Anti-carbohydrate antibodies play a role in the rejection of wild type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig xenografts. We investigated development of these antibodies in infant baboons and humans. Serum samples from infant baboons ($n = 42$) and humans ($n = 42$) were tested by flow cytometry for immunoglobulin M and immunoglobulin G binding to peripheral blood mononuclear cells from WT and GT-KO pigs, and for complement-dependent cytotoxicity. The presence of anti-blood group antibodies was tested in baboon serum. In infant baboons and humans, cytotoxic anti-Galα1,3Gal antibodies develop during the first 3 months, and steadily increase with age, whereas cytotoxic anti-nonGal antibodies are either absent or minimal in the majority of cases throughout the first year of life. Anti-blood group antibodies were not detected before 16 weeks of age. Our data suggest GT-KO pig organ/cell transplants could be carried out in early infancy in the absence of preformed cytotoxic anti-nonGalα1,3Gal antibodies.

Introduction

There is a critical shortage of human organs for transplantation, particularly in infants requiring heart transplants. The Toronto group has utilized hearts from ABO blood group-incompatible donors to overcome the high mortality in infants waiting for a donor heart [1]. They have demonstrated that 'natural' anti-A or -B antibodies (Abs) take several weeks or months to develop in infants and, if an *ABO-incompatible* heart transplant is performed before the development of such Abs, then these Abs *never* develop [2]. They have demonstrated that B-cell tolerance to the specific A and/or B antigen can be achieved in infants who receive immunosuppressive therapy directed only against the T-cell response, which is essentially similar to that used for *ABO-compatible* transplant recipients.

The mechanism by which the primate immune system responds to ABO carbohydrate antigens is believed to be similar to that governing the response to carbohydrate antigens expressed by xenogeneic tissues from genetically distant species, such as swine [3]. This opens the possibility that, if a pig organ is transplanted into an infant before the development of anti-pig Abs, the state of B-cell tolerance to pig carbohydrate antigens may result. The Toronto group's work has therefore suggested a method by which the development of natural anti-pig Abs could be prevented. As an immunosuppressive regimen based on a combination of an anti-CD154 monoclonal Ab and mycophenolate mofetil has been demonstrated to prevent the T cell-dependent elicited Ab response in pig-to-baboon organ and cell transplant models [4–8], pig organ transplantation could theoretically be carried out in infants in the absence of *all* (natural and elicited) Abs directed to pig antigens.

Natural anti-pig Abs develop in humans during the first few months of life, apparently as a response to colonization of the gastrointestinal tract by various microorganisms [9]. Natural Abs against wild-type (WT) pigs include both those directed to Galα1,3Gal (Gal) antigens and those directed to other unidentified antigens, referred to as nonGal; these may be carbohydrate or protein antigens. In contrast, α1,3-galactosyltransferase gene-knockout (GT-KO) pigs express only nonGal antigens. The majority of primate anti-pig Abs are directed to Gal epitopes on the WT pig vascular endothelium and certain other tissues [10–13], and which are also present on various bacteria and viruses [9]. In nonhuman primates and humans, binding of anti-pig Abs, particularly anti-Gal Abs, to transplanted pig organs results in complement activation and hyperacute rejection.

The problem of anti-Gal Abs has been overcome by the generation of pigs homozygous for GT-KO, which renders them incapable of producing the Gal oligosaccharide [14,15]. However, other preformed anti-pig Abs directed to nonGal antigens are present in approximately 50% of adult humans and Old World nonhuman primates [16], and are cytotoxic to pig cells, and may result in a delayed form of Ab-mediated rejection known as acute humoral xenograft rejection [5,6,17]. A method of preventing the development of natural anti-pig Abs would therefore greatly enhance the prospect of successful pig organ transplantation in primates as it would negate the injury caused by such Abs.

The presence of anti-Gal Abs in newborn baboons was initially investigated several years ago [18–23]. Only maternal immunoglobulin G (IgG) was present in the tested serum samples from neonates. Although the transplantation of an unmodified WT pig organ into an untreated baboon during the first few weeks of life rapidly led to the development of elicited anti-Gal IgG that caused AHXR, the absence of preformed anti-Gal immunoglobulin M (IgM) meant that hyperacute rejection did not occur. These studies therefore suggested that the transplantation of a WT pig organ very early in life, in the presence of no, or only low levels of, anti-pig IgM, is associated with an absence of hyperacute rejection.

However, little is known about the rate of development of anti-pig Abs in the first year of life, particularly in regard to anti-nonGal Abs. We have investigated the development of anti-pig Abs, and specifically anti-nonGal Abs, in both infant baboons and humans, and we address the implications that our findings may have for pig organ transplantation into newborns. We hypothesized that infant humans and baboons would not have significant levels of anti-pig Abs in the first few months of life, and that anti-nonGal Abs may increase at a slower rate during the first year of life. We also hypothesized that the pattern of change between infant humans and baboons would correlate such that baboons would be considered a suitable surrogate for humans in preclinical studies.

Materials and methods

Sources of serum

Humans

Serum samples (n = 42) from humans (age <1–64 weeks) were from patients being assessed for a variety of reasons, including cardiac surgical procedures (Loma Linda University, Loma Linda, CA, USA and University of Alberta, Edmonton, Canada). Samples were tested for IgM and IgG binding to GT-KO (n = 42) and WT (n = 33) pig peripheral blood mononuclear cells (PBMC) by flow cytometry, and 41 samples were tested for cytotoxicity.

The Edmonton Health Research Ethics Board required documentation of informed consent prior to acquisition of infant serum samples, and this was accomplished. Because of the following caveat, the Loma Linda Institutional Review Board deemed that the need for consent from parents or legal guardians was unnecessary. Loma Linda human serum samples, obtained for clinical purposes other than this research, were identified and salvaged (before being discarded) by assigned third-party laboratory technicians. Samples were labeled on the basis of donor age alone, before delivery to the principal investigators. Hence, patient identity and right to know would not be violated or traceable. This represents a well-considered difference of opinion between the two human studies review boards, and is not germane to the present study.

Baboons

Serum samples (n = 56) were collected from 42 baboons (age 6–70 weeks) (OUHSC, Oklahoma City, OK, USA). Forty samples were from 26 colony-raised infant baboons,

of which 14 were tested a second time (23 weeks after the first sampling). The other 16 samples were from baboons maintained under specific pathogen-free (SPF) conditions. All initial serum samples were tested for IgM and IgG binding to WT and GT-KO pig PBMC by flow cytometry and for complement-dependent cytotoxicity, but the 14 samples retested after 23 weeks were tested only for cytotoxicity.

All animal care procedures were in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Sources and isolation of porcine PBMC

To detect the presence of anti-pig (Gal + nonGal) and anti-nonGal Abs in these sera, PBMC were collected from the blood of a single WT and a single GT-KO [14] pig, to avoid variability between different WT and GT-KO pig cells. Both were of Large White/Landrace/Duroc cross-breed (Revivicor, Blacksburg, VA, USA). (Several studies in our laboratory have demonstrated that there is little variability in IgM/IgG binding and serum cytotoxicity to PBMC from different pigs from the same source.) The PBMC were used as targets in flow cytometry and cytotoxicity assays. The GT-KO pigs differed from the WT pigs only with regard to the absence of Gal epitopes [14]. Isolation of PBMC was performed by gravity centrifugation, as previously described [16].

Flow cytometry

IgM and IgG binding to WT and GT-KO PBMC was determined by flow cytometry, as previously described [16]. Briefly, isolated PBMC were resuspended in FACS-buffer [PBS containing 1% bovine serum albumin (Gibco, Grand Island, NY, USA) and 0.1% NaN_3] to a final cell concentration of 12.5×10^6 cells/ml. One million PBMC were then removed and incubated with 20 μl of heat-inactivated primate serum (at 56 °C for 30 min), diluted in FACS buffer to 20% serum final concentration, for 30 min at 4 °C. After incubation, the cells were washed twice in 2 ml FACS buffer, vortexed and centrifuged at 700 *g* for 5 min. Cells were then resuspended in 10 μl of 10% goat serum in FACS buffer, to prevent nonspecific binding, and incubated further with 10-μl fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (γ chain-specific) at 1:50 dilution, or with IgM (μ chain-specific) at 1:200 dilution, (Zymed Laboratories, San Francisco, CA, USA) for 30 min in the dark at 4 °C. Antibodies bound to live cells were analyzed using FACScan (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded from the analysis by forward-scatter and propidium iodide staining (10 μl added to each sample prior to FACS analysis). The mean fluorescence intensity (MFI) reading of binding strength correlates with the level of Ab in the serum samples. The high number of samples necessitated the flow cytometry experiments to be carried out in different batches on different days. To correct for differences in isotype controls on different days, we used an index. The MFI reading of the experimental sample was divided by that of the (isotype) control sample for that individual batch. The isotype control consisted of the MFI reading of PBMC stained with FITC IgM and IgG only, without the addition of serum. Whenever the index was >1.1, the sample was considered positive. We classified serum samples to have minimal binding (index 1.1–1.5), mild-to-moderate binding (index 1.5–2.5), or strong binding (index >2.5).

^{51}Chromium-release complement-dependent cytotoxicity assay

The complement-dependent cytotoxicity assay measured serum cytotoxicity of PBMC that were labeled with ^{51}chromium (^{51}Cr). The three components (serum + complement + target cell) were mixed in microtiter-plate wells, and lysis of the target cells was detected by measuring the release of ^{51}Cr radioactivity into the cell supernatant.

Briefly, ^{51}Cr-labeled target cells were prepared from PBMC that were washed with PBS and centrifuged for 4 min at 4 °C. The supernatant was removed, the pellet was resuspended with 40 μl of 10% FBS, and each 1×10^6 PBMC was incubated with ^{51}Cr of 50 μCi for 60 min at 37 °C. Serum samples were incubated for 30 min at 56 °C to inactivate complement. The samples were fourfold serially diluted with mixed leukocyte reaction (MLR) medium [RPMI 1640 media (Gibco) + 10% FBS] to final concentrations of 25%, 6.0%, 1.5%, and 0.3%. As an interplate control, pooled-sensitized human serum was also diluted in this manner and used on all plates. On a round-bottomed 96-well plate, 0.01×10^6 ^{51}Cr-labeled cells (suspended in 80 μl MLR medium) were loaded into each well, and incubated with 80 μl of diluted serum for 30 min at 4 °C. After incubation, the cells were further incubated with 40 μl of 10% rabbit HLA-ABC serum (Sigma, St. Louis, MO, USA), as a source of complement, for 45 min at 37 °C.

Minimal killing controls (100% live cells) were achieved by adding 80-μl MLR medium or complement to wells containing cells without serum. Maximal killing controls (0% live cells) were achieved by adding detergent [5% (v/v)Triton X-100; Sigma] to wells containing cells without serum.

Following incubation, the cells were centrifuged at 35 *g* for 4 min. The supernatant containing ^{51}Cr released from

lyzed cells was harvested using the Macrowell™ Tube Strips collection system (Molecular Devices, Sunnyvale, CA, USA). Cell killing was assessed by reading the ^{51}Cr released from lyzed cells using a gamma irradiation counter (INC Biomedical, Costa Mesa, CA, USA).

Cell killing was calculated using the following formula:

$$\%\,\text{cytotoxicity} = [(A - C)/(B - C)] \times 100,$$

where A equals the count per minute of sample dead cells (+ serum and complement), B equals the count per minute of maximal dead cells (+ detergent), and C equals the count per minute of minimal dead cells (+ complement only).

Complement-dependent cytotoxicity values of the varying serum concentrations (25%, 6.0%, 1.5%, and 0.3%) were calculated, and a curve was generated for each sample. Results were considered acceptable based on the slope of a curve generated by a graded concentration of human control serum that was added to every plate. Lysis of <10% was considered of doubtful significance.

ABO blood typing of baboons by buccal mucosal smear and hemagglutination assay for anti-A/B antibodies in baboon serum

Blood group antigen (ABH) phenotyping of buccal mucosal smears was accomplished in baboons by immunohistochemical staining, and presence of anti-A/B antibodies in baboon serum was determined by hemagglutination assay, as previously described [24,25].

Statistical analysis

Analysis of indirect immunofluorescence intensity was accomplished by CELL QUEST software and converted into MFI by WINMDI software. Statistical analyses were performed using tests for paired data: the Wilcoxon signed rank test for comparison of medians and the McNemar test for comparison of proportions. Correlation of MFI or cytotoxicity with age was assessed by linear regression analysis. Statistics were calculated using PRISM-4 software (Graphpad Software, San Diego, CA, USA) and SPSS 14.0 for Windows (SPSS, Chicago, IL, USA) and P-values were compared with the 95% level of significance.

Results

Binding of infant human and baboon serum IgM and IgG to pig PBMC by flow cytometry

We studied serum samples of infant humans (n = 42) and infant baboons (n = 56) by flow cytometry for IgM and IgG binding to WT and GT-KO PBMC.

Humans

IgM binding to WT PBMC was seen as early as 1 week of age, and binding increased with age, though not significantly (P = 0.073) (Fig. 1a). No or only minimal IgM binding to GT-KO PBMC was seen during the first 30 weeks, and the level of anti-nonGal Abs remained low throughout the first year, with no correlation with age (P = 0.129) (Fig. 1a). Binding of IgM to WT and GT-KO cells is documented in Table 1. IgM binding to both WT and GT-KO PBMC was lower than in baboons (see below), which was consistent with cytotoxicity findings (see below, Fig. 3).

The level of IgG binding to WT PBMC in neonates was high, and decreased during the course of the next few weeks and months, although this fall was not significant (P = 0.381) (Fig. 1b). (These high IgG levels seen soon after birth were likely due to maternal IgG.) In contrast, the level of IgG binding to GT-KO PBMC was low in neonates (not significantly different from isotype control), but increased with age during the first year of life (P = 0.021), although binding remained low.

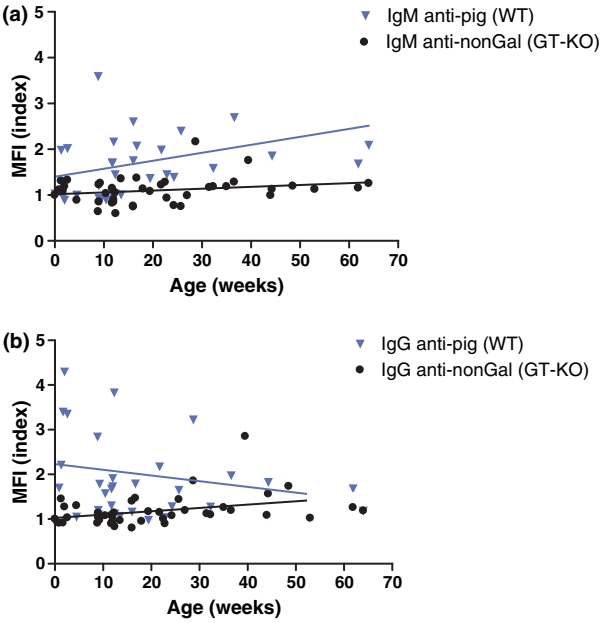

Figure 1 Binding of infant human (n = 42) serum antibodies to wild type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig peripheral blood mononuclear cells (PBMC). [mean fluorescence intensity (MFI) index = mean fluorescence intensity of the serum sample divided by the MFI of the isotype control sample]. (a) Distribution of immunoglobulin M reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, P = 0.073, r = 0.316; versus GT-KO, P = 0.129, r = 0.238). (b) Distribution of immunoglobulin G reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, P = 0.381, r = −0.158; versus GT-KO, P = 0.021, r = 0.356).

Table 1. Strength of binding of infant baboon and human serum* immunoglobulin M (IgM) and immunoglobulin G (IgG) to wild type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig peripheral blood mononuclear cells by flow cytometry.

	Human (n = 42)				Baboon (n = 42)			
	IgM		IgG		IgM		IgG	
	WT	GT-KO	WT	GT-KO	WT	GT-KO	WT	GT-KO
Strength of binding (%)								
No binding	34	48	15	53	5	38	60	98
Minimal	15	48	24	38	28	52	17	0
Mild-to-moderate	30	4	40	7	50	10	21	2
Strong	21	0	21	2	17	0	2	0

*Drawn at various times during the first year of life.

In neonates (age <4 weeks, n = 6), in relation to isotype control, IgG binding to WT PBMC was increased up to approximately four times, while binding to GT-KO PBMC was minimal (Fig. 1b) (P = 0.046). The maternal anti-pig IgG present in the sera of neonates would therefore appear to be almost entirely anti-Gal Ab.

Baboons

There was no or minimal IgM binding to WT PBMC (as defined by MFI-index<1.5, see Materials and methods) documented earlier than 10 weeks of age (n = 4), after which the incidence and extent of binding steadily increased with age (P < 0.001) (Fig. 2a). No or only minimal IgM binding to GT-KO PBMC was seen before 15 weeks, after which there was some increase, but the extent of binding remained low throughout the first year, and there was no correlation with age (P = 0.103) (Fig. 2a). Binding of IgM to WT and GT-KO cells is documented in Table 1.

There was a slight increase in the level of IgG binding to WT PBMC during the first year, though the increase was not significant (P = 0.444) (Fig. 2b). The levels of binding to GT-KO PBMC in most cases remained at the level of the isotype control (MFI-index = 1), and there was no correlation with age (P = 0.740). Binding is documented in Table 1.

Sera from colony-raised baboons (n = 26) and SPF baboons (n = 16) were investigated separately. No significant difference in IgM or IgG binding to either WT or GT-KO pig PBMC was found using sera from these two groups (data not shown), suggesting that anti-pig Ab formation was not influenced by the SPF environment.

Cytotoxicity of infant human and baboon sera to pig PBMC

The same serum samples were tested for complement-dependent cytotoxicity against WT and GT-KO PBMC.

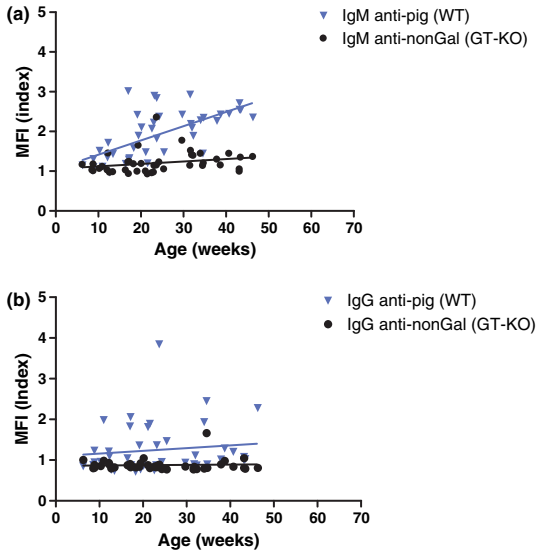

Figure 2 Binding of infant baboon (n = 42) serum antibodies to wild type (WT) and α1,3-galactosyltransferase gene-knockout (GT-KO) pig peripheral blood mononuclear cells (PBMC). [mean fluorescence intensity (MFI) index = mean fluorescence intensity of the serum sample divided by the MFI of the isotype control sample.] (a) Distribution of immunoglobulin M reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, P < 0.001, r = 0.665; versus GT-KO, P = 0.103, r = 0.255). (b) Distribution of immunoglobulin G reactivity against WT and GT-KO PBMC. Correlation of MFI index with age of each group is indicated by a line (versus WT, P = 0.444, r = 0.121; versus GT-KO, P = 0.740, r = 0.053).

All samples showed a titration curve with reduction in lysis with increasing dilution (not shown). To demonstrate correlation of cytotoxicity with age, lysis (=% cytotoxicity) at a serum dilution of 25% is shown in Fig. 3.

Humans

During the first year of life, 39% of infant human sera were cytotoxic (>10% lysis) to PBMC from WT pigs, whereas only 17% were cytotoxic to GT-KO PBMC (P = 0.002) (Fig. 3a). The extent of GT-KO PBMC lysis was significantly less than that of WT PBMC (median percentage of lysis 0% vs. 6%, respectively, P = 0.001). Lysis of WT PBMC increased significantly with age (P = 0.023), but lysis of GT-KO PBMC (i.e. associated with anti-nonGal Abs) did not increase with age (P = 0.803). The median lysis of GT-KO PBMC throughout the entire first year was 0% (range 0–40%).

Baboons

During the first year of life, 67% of infant baboon sera were cytotoxic (defined by >10% lysis) to PBMC from

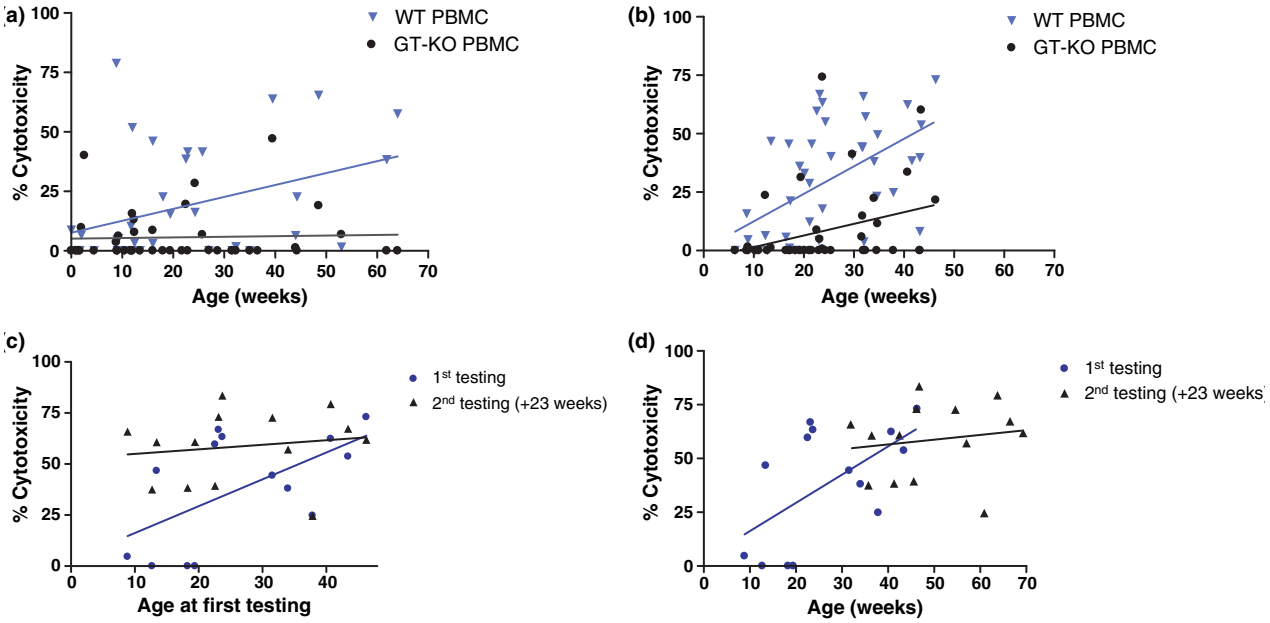

Figure 3 (a) Ability of human serum samples (n = 41) at 25% dilution to cause lysis of peripheral blood mononuclear cells (PBMC) from wild type (WT) or α1,3-galactosyltransferase gene-knockout (GT-KO) pigs. Correlation of complement-dependent cytotoxicity with age is indicated by lines. Significant correlation with age was observed for lysis of WT PBMC (P = 0.023, r = 0.377), but not for lysis of GT-KO PBMC (P = 0.803, r = 0.040). (b) Ability of baboon serum samples (n = 42), tested at 25% dilution, to cause lysis of PBMC from WT or GT-KO pigs. Correlation of complement-dependent cytotoxicity with age is indicated by lines. Significant correlation with age was observed for both lysis of WT PBMC (P < 0.001, r = 0.522) and GT-KO PBMC (P = 0.035, r = 0.330). (c) Ability of baboon serum samples (n = 14), tested at 25% dilution on two occasions (the second 23 weeks after the first), to cause lysis of PBMC from a WT pig. At the second testing, an increase in cytotoxicity of most baboon anti-pig antibodies (Abs) was documented (n = 11). (d) The same data presented in (c) have been plotted to show the correlation of complement-dependent cytotoxicity with age (indicated by lines). At the initial testing, a significant correlation was observed between lysis and age (circles) (P = 0.029, r = 0.582). At the second testing (23 weeks later; triangles), an increase in cytotoxicity of baboon anti-pig Abs (compared with the initial testing) was documented, but increasing lysis did not correlate with increasing age (P = 0.600, r = 0.153).

WT pigs, whereas only 24% of sera were cytotoxic to GT-KO PBMC (P < 0.001). Furthermore, when lysis was detected, the extent of lysis of GT-KO PBMC was generally much less than that of WT PBMC (median percentage of lysis 0% vs. 29%, respectively, P < 0.001). Lysis increased significantly with age against both WT PBMC (P < 0.001) and GT-KO PBMC (P = 0.035) (Fig. 3b). Lysis of GT-KO PBMC of >10% was rarely documented in sera from baboons <22 weeks of age (2 of 22 samples). Importantly, 76% of baboons demonstrated no detectable cytotoxicity (i.e. >10% lysis) to GT-KO PBMC.

Sera from 14 infant baboons of different ages were retested for cytotoxicity against WT PBMC 23 weeks after the first blood draw. Cytotoxic anti-pig Abs had increased in these baboons during this interval (median extent lysis increased from 46% to 61%, P = 0.012) (Fig. 3c and d). Infant baboons that were older at the time of the first blood draw showed less increase in cytotoxic anti-pig Abs, suggesting that most anti-pig Abs are formed early in the first year of life.

Presence of anti-A or -B blood group antibodies in infant baboons

Blood groups of baboons were determined by staining of buccal swabs. Sera from baboons (n = 20) with blood group A or B were then tested for the presence of anti-B or -A blood group Abs, respectively. Sera from baboons with blood group AB were not followed up, as they never develop anti-A or -B Abs. In the sera tested, we did not detect any anti-A or -B Abs before the age of 16 weeks (Fig. 4). Most sera of baboons of 17 weeks and older demonstrated detectable levels of Abs, although, in some cases, absence of Abs was seen up to 23 weeks of age. This suggests that anti-blood group Abs do not develop in baboons until the fourth month of life, and are present in most baboons >5 months of age.

All baboons with anti-blood group Abs demonstrated detectable levels of anti-WT pig IgM, but IgM binding to WT pig cells was also observed in sera without anti-A/B Abs. We found a correlation between the presence of anti-A/B Abs and a higher level of IgM binding to WT

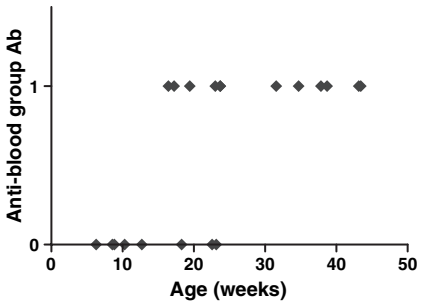

Figure 4 Presence of anti-A or -B blood group antibodies (Abs) in sera of blood group B or A baboons, respectively, of different ages (n = 20). (0 = anti-A or -B Abs were not detected; 1 = anti-A or -B Abs were present).

cells (P = 0.029), but no correlation was found between the presence of anti-A/B Abs and binding of anti-WT IgG (P = 0.357) or of anti-GT-KO IgM (P = 0.136) or anti-GT-KO IgG (P = 0.715).

Discussion

Natural anti-pig Abs develop during infancy [18–23], a finding confirmed in the present study. However, this is the first investigation of the development of anti-nonGal Abs in infant humans and baboons and the first demonstration of the correlation between age and Ab production. Our data suggest that both baboons and humans probably develop cytotoxic anti-pig IgM early during the first year, the level of which steadily increases with age during the first year, but this IgM is largely directed against Gal targets. Cytotoxic anti-nonGal Abs develop later, and remain at a minimal level in the majority of cases (Figs 1–3). This increase in anti-pig Abs with age during the first year is consistent with that of anti-A/B Abs.

As described in adults [16], the incidence and extent of binding of infant human (Fig. 1) and baboon (Fig. 2) serum IgM and IgG to PBMC from GT-KO pigs was significantly less when compared with binding to WT PBMC. The associated lysis of WT cells was higher in baboons than in humans (P = 0.031) (Fig. 3), suggesting that Ab-mediated rejection of a transplanted WT pig organ may be more problematic in the baboon than in the human. The increase in anti-pig cytotoxic Abs was confirmed by sequential measurement of sera from the same baboon (Fig. 3). More importantly, the cytotoxicity of infant human anti-nonGal Abs did not increase with age and was absent in a significant number of cases. This allows a 'window of opportunity' during which a GT-KO pig organ could be transplanted into a human infant without the risk of rejection associated with the presence of natural anti-nonGal cytotoxic Abs. Newborns with congenital heart disease, for whom no human donor heart is available, could be possible candidates for receiving a xenotransplant early in life. As occurs in the case of ABO-incompatible heart allotransplantation in human infants [1,2], B-cell tolerance to carbohydrate antigens may develop when a GT-KO pig heart is transplanted into a primate recipient that has not yet developed anti-nonGal Abs.

However, in contrast to ABO-incompatible allotransplantation, the transplantation of pig hearts might be less successful as cellular components of the innate immune response may be problematic. Furthermore, immune responses to other xenoantigens (against which no natural Abs are present) would need to be suppressed, with the associated risks of immunosuppressive therapy. But, it is not definitively known whether immunosuppressive therapy would need to be continued throughout the life of the recipient. Data from Bailey *et al.* [26,27] suggest that the immune response to an allograft in neonates and infants is rather less vigorous than in later life, and West *et al.* [1] have drawn attention to the relative 'plasticity' of the neonatal immune system. It would be of great clinical interest to study whether, in this age group, the prevention of a T-cell response for a period of time might result in T-cell tolerance.

Although our study indicates that anti-nonGal Ab levels in infant sera are frequently absent or low, the presence of a significant level of natural or elicited anti-nonGal Abs is almost certain to result in Ab-mediated rejection [16,28,29]. The target(s) for these anti-nonGal Abs need to be ascertained [30,31].

Baboons would generally appear to develop anti-A/B Abs at approximately 4 months of life, which is similar to the time of their development in humans [1]. A previous study using adult human and baboon sera showed no statistically significant correlation between the presence of anti-Gal Abs and anti-A/B Abs [32], which is confirmed for IgG Abs by our findings. However, we found a correlation between the presence of anti-A/B Abs and anti-WT IgM in infant baboons, possibly as both levels are known to increase during the first year of life.

There is a discrepancy between the high level of (maternal) anti-pig (Gal) IgG in newborn humans and the relative absence of IgG in newborn baboons. Some or all of this discrepancy is likely to be related to the fact that some human sera were available to us as early as the first week of life, whereas no baboon sera were available until the sixth week. However, the relatively lower levels of IgG seen in the first 3 months in baboons suggest that there may be a difference between the two species in this respect, although maternal IgG has been documented in neonatal baboons previously [20].

In summary, our data suggest that GT-KO organ transplants could be carried out in early infancy in human recipients in the complete or relative absence of cytotoxic anti-nonGal Abs. If immunosuppressive therapy were administered to suppress a T cell-mediated elicited Ab response, xenograft rejection resulting from anti-nonGal Abs would be prevented (although other potential problems may still arise [33]). Based on the clinical experience of heart allotransplantation in infant humans across the ABO barrier, it is possible that B-cell tolerance to pig carbohydrate antigens might develop. Future studies should focus on testing this *in vivo*, by implanting tissue/organs from GT-KO pigs into infant baboons.

Acknowledgements

The authors thank Lisa Goldberg and Mark Yazer, MD of the Institute for Transfusion Medicine in Pittsburgh, PA, for technical help and advice. Pleunie Rood was supported, in part, by a grant from the Prof. Michaël van Vloten Fund. Research in our laboratory was supported by NIH grant #U01 AI068642 and by a Sponsored Research Agreement between the University of Pittsburgh and Revivicor, Inc.

Authorship

PPMR contributed to the design of the study, performed flowcytometry tests, cytotoxicity assays and data analyses, and contributed in writing of the manuscript. HCT performed a significant number of cytotoxicity assays. HH supervised flowcytometry and cytotoxicity experiments. CL performed blood group antibody tests by buccal swab staining and serum antibody testing. ME contributed in establishing the blood group antibody tests. YJL contributed in establishing the cytotoxicity assay. DJW performed parts of the statistical analyses. JB performed parts of the blood group antibody testing. DA contributed to the design of the study, provided pig blood, provided blood samples from infants, contributed to the presentation of the data and writing of the manuscript. JNMI contributed to the design of the study and writing of the manuscript. RFW contributed to the design of the study, provided blood samples from baboon infants, contributed to the presentation of the data and writing of the manuscript. RM contributed to the design of the study, provided blood samples from infant patients, and contributed to the presentation of the data and writing of the manuscript. LB contributed to the design of the study, provided blood samples from infants, contributed to the presentation of the data and writing of the manuscript. DKCC contributed to the design of the study, gave supervision to all experiments, provided blood samples from infants, and contributed to the presentation of the data and writing of the manuscript.

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