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Increase in Anti-Gal IgM Level is Associated With Early Graft Failure in Intraportal Porcine Islet Xenotransplantation

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Background: Anti-Gal is a major antibody induced in non-human primates (NHPs) after xenotransplantation. To understand the mechanism of graft rejection, we investigated the association between anti-Gal responses and graft failure in NHP recipients of porcine islet transplantation (PITx).

Methods: Intraportal PITx was performed in 35 diabetic NHPs, and graft function was monitored. Early graft failure (EGF) was defined as loss of graft function within a month after PITx. Seven, 19, nine NHPs received immunosuppression (IS) without CD40 pathway blockade (Group I), with anti-CD154 (Group II), and with anti-CD40 (Group III), respectively. The anti-Gal levels on day 0 and day 7 of PITx were measured by ELISA.

Results: The frequency of EGF was significantly lower in Group II (26.3%) than in Group I (100%, P=0.0012) and Group III (77.8%, P=0.0166). While levels of anti-Gal IgG in Group I and anti-Gal IgM in Group III increased on day 7 compared with day 0 (P=0.0156 and 0.0273), there was no increase in either on day 7 in Group II. The ratio of anti-Gal IgM or IgG level on day 7 to that on day 0 (Ratio7/0) was significantly higher in recipients with EGF than without EGF (P=0.0009 and 0.0027). ROC curve analysis of anti-Gal IgM Ratio7/0 revealed an area under the curve of 0.789 (P=0.0003).

Conclusions: IS with anti-CD154 suppressed anti-Gal responses and prevented EGF in PITx. Anti-Gal IgM Ratio7/0, being associated with EGF, is a predictive marker for EGF.

Key Words: Pig, Non-human primate, Islets, Xenotransplantation, Antibody, Gal, Early graft failure

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INTRODUCTION

Islet transplantation is a useful therapeutic modality for patients with type 1 diabetes [1, 2]; however, a shortage of donor organs impedes the widespread application of islet transplantation [3]. Porcine islets have been proposed as a readily available and conceptually unlimited alternative source [4], and long-term glucose control has been achieved in diabetic non-human primate (NHP) recipients of intraportal porcine islet transplantation (PITx) with the use of CD40 costimulation pathway blockade [5-9]. However, the reported graft survival is not highly reproducible.

PITx in a human or NHP recipient elicits humoral responses against porcine antigens, including Galα1,3Gal (Gal) and non-Gal antigens [10]. Gal is a carbohydrate antigen, expressed universally in most species including bacteria and fungi, but not in humans or NHPs [11]. Anti-Gal is the most abundant natural

antibody in humans, which is found as IgM, and to a lesser extent as IgG and IgA; it is continuously produced in response to antigenic stimulation by common gut bacteria [11, 12]. Use of Gal-deficient porcine organ significantly prevents graft rejection in organ xenotransplantation [13, 14], but transplantation of Gal-deficient porcine islets does not improve graft survival in PITx [15].

CD40 signaling on B cells via interaction with CD154 expressed on T cells is critical for B cell survival and proliferation, antibody production and isotype switching, germinal center formation, memory generation, and cytokine production [16]. Anti-CD154 or anti-CD40 antibodies targeting the CD40 costimulation pathway efficiently suppress humoral responses in the recipient of a xenotransplantation [5, 9, 17-19]. We previously reported that an immunosuppression (IS) regimen involving the use of anti-CD154 suppresses the induction of anti-non-Gal and anti-Gal antibodies and prolongs graft survival for up to a year in NHP recipients of PITx [8, 10]. In contrast, a similar IS regimen involving anti-CD40 suppressed xenoreactive IgG responses after PITx as well, but failed to sustain the graft function for longer than a month [10]. Thus, suppression of humoral responses against xenoantigens appears to be essential but insufficient for sustained graft survival in PITx. The exact mechanism underlying early graft rejection in recipients with anti-CD40 IS is not clearly defined. Considering humoral responses are brought about by cross-talk with other innate and adaptive immune cells following contact with the antigen [20-22], precise characterization and measurement of xenoreactive antibodies after transplantation may provide useful information on graft rejection. Thus, we measured the levels of anti-Gal IgG and IgM prior to and on day 7 of PITx and investigated the association of antibody responses with the graft survival in the NHP recipients of PITx. This study demonstrates that the ratio of the anti-Gal antibody level on day 7 to that on day 0 of PITx is significantly associated with the loss of graft function within a month after PITx.

METHODS

1. Intraportal PITx in rhesus monkeys

All data on animal experiments, the use of IS agents, and the survival of the grafts were retrospectively collected from the archival database of our research group and have partly been previously reported [8, 10]. Preparation of the porcine islets and intraportal transplantation were performed as previously described [8]. All animals used in this study were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals; the primate study protocols were approved by the Institutional Animal Care and Use Committee at Seoul National University Hospital, Seoul, Korea. Briefly, islets were isolated by using the modified Ricordi method from wild-type Seoul National University miniature adult pigs [23, 24]. Isolated porcine islets were infused via the portal vein into streptozotocin-induced diabetic rhesus monkeys receiving induction IS. Data for a total of 35 transplantations in naïve rhesus monkeys were collected; data of repeat transplantations or transplantations into recipients with previous exposure to porcine tissues were excluded. The induction IS regimens are summarized in Table 1: seven recipients received various induction IS regimens without CD40 pathway blockade (Group I) and 28 recipients received induction IS with anti-CD154 (n=19, Group II) or anti-CD40 (n=9, Group III). The dose of IS regimen and route of administration were described in our previous studies [8, 10]. Blood samples were drawn in EDTA tubes from each recipient prior to the transplantation (day 0) and a week after transplantation (day 7 +/- 2 days). Aliquots of plasma samples were stored at -70°C until they were used. The survival of the graft was determined as the time at which the recipient did not maintain euglycemia without exogenous insulin at a dose similar to the pre-PITx

Table 1. Frequencies of EGF in 35 recipients after porcine islet transplantation according to induction immunosuppression regimen

Group	Induction immunosuppression	N	EGF
Group I no CD40 pathway blockade (N=7)	Tacrolimus, leflunomide, MMF, basiliximab, rituximab	1	7 (100%)
	Campath, tacrolimus, leflunomide, MMF	1	
	ATG, bortezomib, leflunomide, MMF	1	
	ATG, bortezomib	1	
	Anti-ICAM1, bortezomib, rituximab	1	
	Anti-ICAM1	1	
	Anti-ICAM1, bortezomib, leflunomide	1	
Group II anti-CD154 (N=19)	Anti-CD154, sirolimus, anti-ICAM1	10	5 (26.3%)*
	Anti-CD154, sirolimus, ATG	7	
	Anti-CD154, sirolimus	2	
Group III anti-CD40 (N=9)	Anti-CD40, sirolimus, ATG	2	7 (77.8%)
	Anti-CD40, sirolimus, CTLA4-Ig	1	
	Anti-CD40, sirolimus, ATG, CTLA4-Ig	2	
	Anti-CD40, sirolimus, tacrolimus	1	
	Anti-CD40, sirolimus, tacrolimus, ATG	3	

*Fisher's exact test; vs. Group I, $P=0.0012$; vs. Group III, $P=0.0166$.
Abbreviations: Anti-ICAM1, anti-human intercellular adhesion molecule 1 antibody; ATG, anti-thymocyte globulin; CTLA4-Ig, CTLA4-immunoglobulin; EGF, early graft failure; MMF, mycophenolate mofetil.

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requirement. Graft survival of less than a month regardless of the cause was defined as early graft failure (EGF).

2. Anti-Gal IgG and IgM measurements

The levels of anti-Gal IgG and IgM antibodies were measured by using an in-house ELISA as previously described [10]. In brief, each well was coated with 100 μL of Galα1-3Galβ1-4GlcNAc-human albumin (5 μg/mL; GlycoTech, Gaithersburg, MD, USA) and blocked with 1% human albumin (Green Cross Corp., Yongin, Korea) diluted in phosphate buffered saline (PBS). Recipient monkey plasma (100 μL) diluted 1:50 (for anti-Gal IgG) or 1:100 (for anti-Gal IgM) in PBS containing 0.1% human albumin was added into each well in duplicate and incubated at 37°C for 30 min. Subsequently, antibody binding was detected with peroxidase-conjugated anti-human IgG or anti-human IgM (Sigma-Aldrich, St. Louis, MO, USA) and subsequent color reactions. Serial dilutions of a selected rhesus monkey plasma (as a calibrator), designated as 2,809 artificial units (AU)/mL of anti-Gal IgG and 5,610 AU/mL of anti-Gal IgM, were tested in parallel. A mean absorbance of the sample was compared with that of the calibrator, and each antibody level of the sample was calculated from the calibration curve. Positive control plasma was simultaneously tested in each run, and the cumulative coefficient of variation of control plasma for 90 independent assays was 4.53% at the mean concentration of 98 AU/mL for anti-Gal IgG and 6.33% at the mean concentration of 1,169 AU/mL for anti-Gal IgM. The calibrator and control plasma were obtained from rhesus monkeys after repeated sensitization with porcine islets. The reference range of anti-Gal IgG and IgM calculated from 45 naïve rhesus monkeys was less than 121 AU/mL and less than 625 AU/mL, respectively.

3. Statistical analysis

Antibody levels were expressed as the median (range) of a group. Differences in antibody levels between day 0 and day 7 were compared by the Wilcoxon signed rank test, and differences between the groups were compared by the Mann-Whitney U test. Differences in the frequency of EGF between the groups were compared by the Fisher's exact test. The predictive performance of antibody ratio for EGF was evaluated by ROC curve analysis. A *P* value less than 0.05 was considered significant.

RESULTS

1. Graft survival after PITx according to induction IS regimen

The survival of 35 NHP recipients of PITx varied from 3 day to longer than 750 day (Fig. 1). Among them, 19 recipients (54.3%) lost graft function within a month, defined as EGF. When we compared the frequency of EGF according to the type of induction IS agent used (Table 1), the frequency of EGF in Group II (26.3%) was significantly lower than that in Group I (100%, $P=0.0012$) or Group III (77.8%, $P=0.0166$).

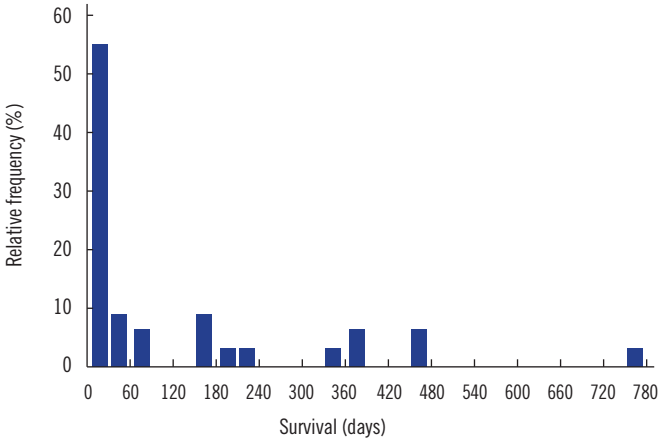

Fig. 1. Histogram of graft survival in 35 non-human primate recipients after porcine islet transplantation.

2. Changes in anti-Gal IgG and IgM levels after PITx according to induction IS regimen

When we compared the levels of anti-Gal between day 0 and day 7 in each PITx recipient, the levels of anti-Gal IgM and IgG increased on day 7 of PITx compared with that on day 0 in each recipient in Group I, although only the increase in the level of anti-Gal IgG was significant (506 vs. 285 AU/mL for IgM, $P=0.0781$; 63 vs. 8 AU/mL for IgG, $P=0.0156$; Table 2). In the recipients of Group II, the level of anti-Gal IgM did not change ($P=0.3955$), and the level of anti-Gal IgG decreased on day 7 (11 vs. 12 AU/mL, $P=0.0361$), indicating a suppression of anti-Gal IgM and IgG responses by anti-CD154. In contrast, the recipients of Group III showed an increase in the level of anti-Gal IgM on day 7 compared with that on day 0 (472 vs. 332 AU/mL, $P=0.0273$) but no change in the level of anti-Gal IgG, suggesting a suppression only of the anti-Gal IgG response by anti-CD40.

When the ratio of the level of anti-Gal antibody on day 7 to that on day 0 (Ratio7/0) was compared between three IS groups (Table 2), the anti-Gal IgM Ratio7/0 in the recipients of Group II (0.92) tended to be or was significantly lower than those in the recipients of Group I (1.78, $P=0.0603$) and Group III (1.52, $P=0.0208$). The values of anti-Gal IgG Ratio7/0 in Group II (0.75) and Group III (1.01) were not statistically different from

Table 2. The plasma levels of anti-Gal antibodies in the recipients of porcine islet transplantation according to induction immunosuppression regimen

Anti-Gal		Group I no CD40 pathway blockade (N=7)	Group II anti-CD154 (N=19)	Group III anti-CD40 (N=9)
IgM, median (range)	Day 0, AU/mL	285 (90-6,926)	363 (42-1,135)	332 (151-502)
	Day 7, AU/mL	506 (57-12,699)	413 (41-2,373)	472 (144-983)
	Ratio7/0	1.78 (0.63-2.03)	0.92† (0.36-12.62)	1.52 (0.84-2.23)
	*P**, day 0 vs. day 7	0.0781	0.3955	0.0273
IgG, median (range)	Day 0, AU/mL	8 (3-13,955)	12 (3-48)	6 (1-228)
	Day 7, AU/mL	63 (4-46,480)	11 (1-39)	13 (2-230)
	Ratio7/0	3.33 (1.25-62.18)	0.75‡ (0.31-5.67)	1.01§ (0.5-6.5)
	*P**, day 0 vs. day 7	0.0156	0.0361	0.5469

*Wilcoxon signed rank test; †Mann-Whitney test; vs. Group I, $P=0.0603$; vs. Group III, $P=0.0208$; ‡Mann-Whitney test; vs. Group I, $P=0.0013$; §Mann-Whitney test; vs. Group I, $P=0.0229$.
Abbreviations: AU, artificial units; Ratio7/0, ratio of the antibody level on day 7 to that on day 0.

Table 3. The relationship of anti-Gal antibody levels with occurrence of EGF in the recipients of porcine islet transplantation

Anti-Gal		No EGF (N=16)	EGF (N=19)	*P*†, no EGF vs. EGF
IgM, median (range)	Day 0, AU/mL	399 (42-1,135)	285 (90-6,926)	0.3625
	Day 7, AU/mL	439 (41-566)	456 (57-12,699)	0.2465
	Ratio7/0	0.85 (0.36-2.17)	1.43 (0.63-12.62)	0.0009
	*P**, day 0 vs. day 7	0.1297	0.0006	
IgG, median (range)	Day 0, AU/mL	15 (3-48)	7 (1-13,955)	0.1907
	Day 7, AU/mL	11 (3-39)	15 (1-46,480)	0.3043
	Ratio7/0	0.74 (0.31-1.75)	1.33 (0.33-62.18)	0.0027
	*P**, day 0 vs. day 7	0.0092	0.0237	

*Wilcoxon signed rank test; †Mann-Whitney test.
Abbreviations: AU, artificial units; EGF, early graft failure; Ratio7/0, ratio of the antibody level on day 7 to that on day 0.

each other but were significantly lower than that in Group I (3.33, $P=0.0013$ and 0.0229).

3. Relationship between the levels of anti-Gal antibodies and EGF

We next compared the levels of anti-Gal antibodies of the recipients of PITx with and without EGF. There was no difference between these two groups of recipients in the levels of anti-Gal, either IgM or IgG, on day 0 ($P=0.3625$ and 0.1907, respectively) and in those on day 7 ($P=0.2465$ and 0.3043, respectively) of PITx (Table 3). However, when we compared the paired levels of anti-Gal antibodies between day 7 and day 0 in each PITx recipient, the level of anti-Gal IgG significantly decreased on day 7 and the level of anti-Gal IgM did not change ($P=0.0092$ and 0.1297, respectively) in the recipients without EGF, but the levels of anti-Gal IgM and IgG significantly increased on day 7 in the recipients with EGF ($P=0.0006$ and 0.0237, respectively). Accordingly, the values of anti-Gal IgM Ratio7/0 and IgG Ratio7/0 were significantly higher in the recipients with EGF than in the recipients without EGF (IgM Ratio7/0, 1.52 vs. 0.85, $P=0.0009$; IgG Ratio7/0, 1.33 vs. 0.74, $P=0.0027$), suggesting a strong association between relative increase of anti-Gal responses on day 7 compared with day 0 and development of EGF in each recipient of PITx.

When the predictive performances of the anti-Gal Ratio7/0 for EGF were analyzed by ROC curve, the area under the ROC curve (AUC) of anti-Gal IgM and IgG Ratio7/0 was 0.789 and 0.799 ($P=0.0003$ and 0.0002, respectively; Fig. 2A) in the analysis of the data from all recipients, and 0.816 and 0.703 ($P=0.0001$ and 0.0783, respectively; Fig. 2B) in the analysis of the data from the recipients in Group II and Group III, revealing a significant performance of anti-Gal IgM Ratio7/0 for the prediction of EGF.

DISCUSSION

This is the first report demonstrating the association between humoral responses and graft outcome in NHP recipients of PITx. Anti-Gal antibodies are frequently induced in NHP recipients of PITx. However, because islet graft rejection precedes antibody induction and the use of Gal-deficient porcine islets delay but not prevent graft failure [15], antibody responses in

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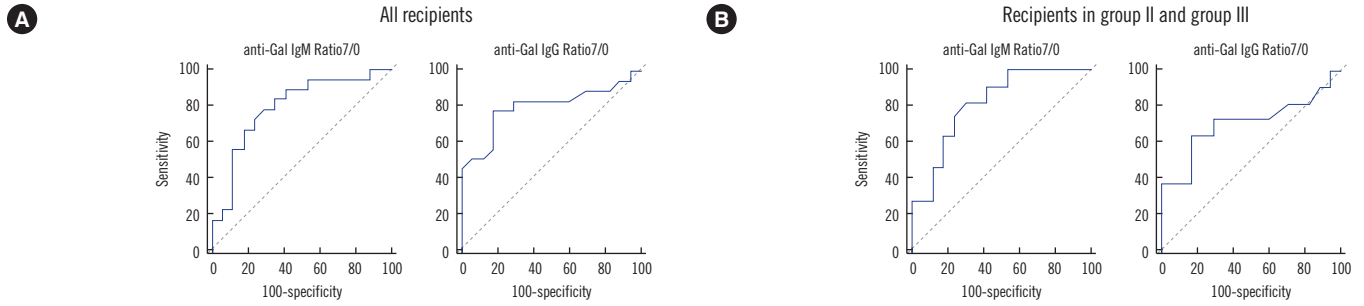

	All recipients		Recipients in group II and group III	
	anti-Gal IgM Ratio7/0	anti-Gal IgG Ratio7/0	anti-Gal IgM Ratio7/0	anti-Gal IgG Ratio7/0
AUC (95% CI)	0.789 (0.618-0.908)	0.799 (0.630-0.915)	0.816 (0.624-0.936)	0.703 (0.502-0.860)
P	0.0003	0.0002	0.0001	0.0783
Criterion	>1.08	>0.98	>1.04	>0.98
Sensitivity, % (95% CI)	82.4 (55.6-96.2)	77.8 (52.4-93.6)	81.8 (48.2-97.7)	63.6 (30.8-89.1)
Specificity, % (95% CI)	66.7 (41.0-86.7)	82.4 (56.6-96.2)	70.6 (44.0-89.7)	82.4 (56.6-96.2)

Fig. 2. Receiver operating characteristic (ROC) curves of anti-Gal IgM Ratio7/0 and IgG Ratio7/0 for the prediction of early graft failure (loss of graft function within a month) in porcine islet transplantation (PITx) analyzed from the data of all recipients (n=35, A) and from the data of the recipients receiving CD40 pathway blockade (n=28, B). Using an in-house ELISA, the levels of anti-Gal IgG and IgM were quantitatively measured in the plasma samples obtained from the rhesus monkey recipients prior to PITx (day 0) and on day 7 (±2) of PITx. The values of anti-Gal IgM Ratio7/0 and IgG Ratio7/0 for each recipient were calculated from the equation: (antibody level on day 7)/(antibody level on day 0). The area under the ROC curve (AUC) with *P* value, sensitivity, and specificity at a given optimal criterion are summarized.
Abbreviation: CI, confidence interval.

PITx have not been a primary concern. In this study, an increased value of anti-Gal antibody Ratio7/0 was associated with early loss of graft function in PITx. Furthermore, we demonstrated that anti-CD40 and anti-CD154 have different effects on anti-Gal IgG and IgM responses.

The costimulatory pathway of CD40-CD154 interaction is essential for activation of T cells against antigens [25, 26] and plays an important role in mounting T cell-dependent humoral responses [27, 28]. Consistent with previous studies [5, 6, 19, 29], the use of anti-CD154 in this study suppressed anti-Gal antibody responses and reduced the frequency of EGF in NHP recipients of adult PITx. Unfortunately, anti-CD154 is not clinically applicable because of thromboembolic complications [30]; therefore, anti-CD40, which blocks CD40-CD154 interaction in an alternative way, has been used in place of anti-CD154. However, as shown in this study and previously [10], anti-CD40 is not as effective as anti-CD154 in prolonging graft survival. Interestingly, anti-CD154 suppressed the induction of both anti-Gal IgG and IgM on day 7 of PITx, whereas anti-CD40 only suppressed an increase of anti-Gal IgG.

In this study, the levels of anti-Gal IgM and IgG increased significantly on day 7 compared with those on day 0 in each PITx recipient with EGF, and anti-Gal IgM and IgG Ratio7/0 significantly correlated with the occurrence of EGF. However, there was no difference in the levels of anti-Gal, either IgM or IgG, on day 0 and day 7 of PITx between the recipients with and without EGF. These results demonstrate that EGF in the recipients receiving IS are not caused by antibody-mediated cytotoxicity and that relatively increased antibody responses on day 7 compared with day 0 in the PITx recipients are epiphenomena reflecting uncontrolled activation of immune cells resulting in EGF, as previously suggested in other reports [10, 15, 31].

Thomson *et al*. [15] reported induction of anti-Gal IgM responses in Gal-deficient xenoislet recipients, indicating that the anti-Gal IgM response is induced by non-specific inflammation independently of the exposure to porcine Gal antigen. Diverse innate immune cells like natural killer (NK) cells, NK T cells, and neutrophils are reported to help B cells produce antibodies against carbohydrate antigens like Gal [22, 32-34]. Taken together, it appears that increased anti-Gal IgM Ratio7/0 may be linked to the activation of innate immune cells following PITx, which mediates graft damage. Recently, Giovannoi *et al*. [35] reported the enhancement of islet engraftment and achievement of long-term islet allograft survival by Toll-like receptor 4 blockade in murine islet transplantation models, supporting our speculation of the association between the activation of innate immune cells and EGF in PITx recipients.

It is not clear why anti-CD40 does not prevent EGF while anti-CD154 does, considering both inhibit CD40-CD154 pathway signaling. While CD40 is expressed on antigen presenting cells such as endothelial cells, B cells, monocytes, and dendritic cells, CD154 is expressed on cytotoxic effector cells such as activated CD4+ and CD8+ T cells, NK cells, basophils, and eosinophils [36]. The CD40-CD154 interaction delivers bidirectional

signals: forward signaling for the activation of antigen presenting cells and reverse signaling for the stimulation of T cells and innate NK cells [37]. The close association between anti-CD154 IS and the prevention of EGF allows us to hypothesize an additional inhibitory effect of anti-CD154 on reverse signaling of CD154 in innate effector cells attacking porcine islet grafts.

This study was not designed to clarify the precise mechanism of graft rejection nor to prove the effect of each IS regimen on graft survival. However, our study with NHP recipients of PITx demonstrates that lower values of anti-Gal IgM Ratio7/0 are associated with anti-CD154 induction IS and prevention of EGF. The anti-Gal IgM Ratio7/0 may be useful to predict subsequent development of EGF in NHP recipients of PITx .

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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