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Is there a Correlation between Anti-Pig Antibody Levels in Humans and Geographic Location During Childhood?

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Abstract

Background—An initial observation suggested high levels of anti-pig antibodies in healthy humans who had spent their childhood in the Middle-East. We tested larger cohorts to determine whether anti-pig antibody levels correlated with the geographic location in which the subject spent his/her childhood, as this might have implications for clinical trials of xenotransplantation.

Methods—Anti-pig IgM and IgG levels (by flow cytometry using PBMC from wild-type [WT] and $\alpha 1$, 3-galactosyltransferase gene-knockout [GTKO] pigs), and anti-Gal IgM and IgG levels (by ELISA) were measured in 75 volunteers. Comparisons of antibody levels were also made on the basis of subject age, gender, ABO blood group, diet, and history of vaccination.

Results—Antibody binding to GTKO pig cells was less than to WT cells. There was a reduction in anti-pig IgM and anti-Gal IgM, but a slight increase in anti-non Gal IgG, with age. Women had higher levels of anti-Gal IgM than men. Blood group A subjects had higher levels of anti-pig IgM and IgG than those of group AB. Diet had no influence on antibody levels. Typhoid or MMR vaccination was associated with lower anti-nonGal IgG or anti-Gal IgG, respectively, whereas influenza vaccination was associated with higher anti-nonGal IgG. There were some significant variations in antibody levels associated with location during childhood, with subjects from the middle-east demonstrating higher anti-nonGal IgG and anti-Gal IgG.

Conclusion—Clinical trials of xenotransplantation may be influenced by various factors, including the geographic location of the recipient during childhood, possibly associated with exposure to different microorganisms.

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Keywords

ABO blood groups; anti-pig antibodies; diet; gender; geography; vaccines

Introduction

Blood is drawn from most members of our laboratory for use in various immunological assays. The volunteers of this blood are from a wide variety of geographic and ethnic backgrounds. Although all subjects have lived in the United States for a number of months or years, and in a few cases for a prolonged period of time (>20 years), our initial observation suggested high levels of anti-pig antibodies in subjects who had spent their formative years (childhood and youth) in Middle-Eastern countries.

We hypothesized that this variation might be associated with exposure to different viruses, bacteria, fungi, and/or parasites during infancy and childhood (1,2,3,4,5,6,7,8). We therefore determined to test larger cohorts of subjects from various geographic backgrounds to ascertain whether the level of anti-pig antibody correlated with the geographic location in which the subject spent his or her formative years. If statistical differences in antibody levels could be confirmed, this might have implications for clinical trials of xenotransplantation in the future. The geographic areas as we defined them are indicated in Table 1.

We also investigated the influence on anti-pig antibody levels of the subjects' age, gender, diet, and history of vaccination.

Results

Antibody Binding To Wt And Gtko Pig PbmC

There was significantly less IgM and IgG binding to GTKO pPBMC than to WT pPBMC (Figure 1). Mean binding to GTKO pPBMC (i.e., to nonGal antigens) was approximately one-third of mean binding to WT pPBMC (i.e., Gal+nonGal antigens), suggesting that perhaps two-thirds of all anti-pig antibody are directed to Gal antigens. Correlation between IgM and IgG binding to WT pPBMC was not significant (Supplemental figure 1A, left), but there was a positive correlation between IgM and IgG binding to GTKO pPBMC ($p < 0.001$) (Supplemental figure 1A, right). There was no correlation between anti-Gal IgM and IgG levels by ELISA (Supplemental figure 1B).

Age

The average age of the human subjects enrolled in the study was 35.7 years (22 to 71 years). With increasing age, there was a significant decrease in anti-pig IgM binding to WT pPBMC ($p < 0.001$) and in anti-Gal IgM levels ($p < 0.01$) (Figure 2). There was a positive, though weak, correlation between age and anti-nonGal IgG levels ($p < 0.05$), indicating an increase in anti-nonGal antibodies with advancing age. There was no significant correlation between anti-pig IgG, anti-nonGal IgM or anti-Gal IgG with age.

Gender

There was a significant difference in the level of anti-Gal IgM ($p < 0.05$), but not anti-Gal IgG, between males and females, the levels in females being higher (Supplemental table 1), while male and female distribution with respect to age was similar (mean: males 36.4 years; females 35.9 years).

ABO Blood Group

Anti-pig IgM and IgG levels were higher in subjects of group A than in those of group AB (Supplemental table 2), but there were no other significant differences.

Diet

There was no significant difference in anti-pig antibody levels between subjects who included pork in the diet and those who did not (Supplemental table 3).

Vaccinations

The numbers of subjects who had *not* received vaccines were low (Supplemental table 4). There was no significant difference in anti-pig antibody levels between the subjects who had received vaccines for yellow fever, *Hemophilus influenzae* B, *Bacillus Calmette-Guerin* (BCG), hepatitis B, or poliomyelitis, and those who had not. Those who had received influenza vaccine had higher levels of anti-non Gal IgG than those who had not ($p<0.05$). Those who had received typhoid ($p<0.01$) or measles-mumps-rubella ($p<0.05$) vaccines had lower levels of anti-Gal IgG than those who had not.

Differences in anti-pig antibody levels related to geographic location during childhood

There were significantly higher levels of anti-pig IgM in subjects from Japan than in those from Europe, South America, and South East Asia ($p<0.05$) (Table 2 and Figure 3), and in subjects from South Asia than from Europe and South East Asia ($p<0.05$). There were significantly higher levels of anti-nonGal IgG in subjects raised in Middle-Eastern countries than in those raised in Europe, North and South America, and South and South East Asia ($p<0.05$). Those from the Middle East also had higher levels of anti-Gal IgG than those from all other regions ($p<0.01$), except South East Asia.

Discussion

Natural (or preformed) antibodies play a role in innate immunity and provide an initial defense against pathogens (9)(10). The most abundant natural antibodies in human serum are anti-Gal antibodies (1-4% of circulating immunoglobulins) (11,12,13,14) which are of IgM, IgG, IgA, and IgD isotypes (7,14,15). They are believed to develop during infancy (1,16,17) as a response to colonization of the gastro-intestinal tract by viruses, microorganisms, and parasites (1).

The natural antibodies produced by the B-1 subset of B lymphocytes usually increase or remain constant throughout life (18), although in the present study there was a decline in anti-pig IgM binding (to WT pPBMC) and anti-Gal IgM level, and a slight increase in binding of anti-nonGal IgG (to GTKO pPBMC) with advancing age (Figure 2). Chao et al (19) observed that, not only was there a steady increase in anti-pig antibodies during infancy and childhood, but there was a significant decline in anti-pig IgM and serum cytotoxicity in subjects >65 years in age.

These observations differ to some extent from our recent findings relating to anti-Gal antibody levels in GTKO pigs (20). After an initial rise during the first three months of life, the level of anti-Gal IgM remained stable until the second year of life, when it began to decline (though follow-up did not extend beyond three years of age). In contrast, anti-Gal IgG fell significantly after the first six months, but showed some increase during the second year.

Natural antibodies form a barrier to successful xenotransplantation, though the transplantation of organs and cells from genetically-engineered pigs that do not express Gal

(GTKO pigs) has largely prevented hyperacute rejection (21,22). Nevertheless, antibodies directed against nonGal antigens on pig vascular endothelial cells remain problematic (23,24,25,26). The targets on pig cells for preformed anti-nonGal antibodies remain largely unknown (12,13,24,27,28,29,30,31), though expression of N-glycolylneuraminic acid antigens on pig cells will be important when clinical xenotransplantation is undertaken (32,33), and could have been playing a role in the present study as most, if not all, humans have antibodies directed to these antigens.

In the present study, the level of anti-Gal IgM was significantly higher in females than in males (Supplemental Table 1), correlating to some extent with the report of Buonomano et al. (34). Possible reasons for this are speculative, but may be associated with the higher incidence of autoimmune disorders in women. Chao et al (19), however, did not find that gender influenced levels significantly.

Others have reported that subjects with blood group A have higher levels of anti-Gal IgG than those with blood group B, possibly related to the structural similarity between Gal and B antigens (35,36,37); in subjects with the A blood group, the majority of anti-B reactivity results from anti-Gal antibodies capable of recognizing both Gal and B epitopes (35). However, in the present study, although subjects with group A had higher levels of anti-pig IgM and IgG than those with group AB, we found no significant difference between A and B (Supplemental Table 2). The relatively small numbers in each group may have influenced the results.

We found no significant difference in any IgM and IgG levels between subjects who consumed pork and those who did not (Supplemental table 3). Studying a Taiwanese population, Chao et al (19) also found no correlation between diet and anti-pig antibody levels; vegetarians had a slightly higher (though not significant) level of anti-pig IgM and greater serum cytotoxicity than meat-eating subjects. It has been demonstrated that, following immunization with pig PBMC, rats (fed on pig PBMC) have decreased cell-mediated immunity and T_H1-based antibody production (involved in complement-based immunity) (38). The decrease in type 1 antibodies is offset by an increase in type 2 antibodies with no change in total antibody level (38). A possible role for environmental exposure (pork consumption) has been suggested in the development of primed T cell memory against swine antigens (39).

Although in the present study some minor differences in antibody levels were found between those exposed to typhoid, influenza, or MMR vaccines and those not (Supplemental Table 4), the relevance of these observations is uncertain.

Our study was primarily undertaken to determine whether exposure to pathogens (that might express Gal and/or nonGal antigens) that might differ between geographic locations during childhood (<18 years of age) might be associated with different levels of anti-pig, anti-Gal, and/or anti-nonGal antibodies in adults from those regions. If significant differences in levels were observed, this might have implications for clinical trials of pig organ or cell xenotransplantation. The only statistical differences in antibody levels between subjects from the different geographic regions we detected were related to (i) higher anti-pig IgM in those from Japan and, to a lesser extent, those from South Asia, (ii) higher anti-nonGal IgG and higher anti-Gal IgG in those from the Middle East than those from most other (but not all) geographic regions (Table 2 and Figure 3). This suggests that the microorganisms to which a subject is exposed during childhood (or other factors) may possibly influence anti-pig antibody levels throughout life.

There are several limitations to this study, including (i) the relatively small numbers of subjects tested, (ii) the fact that the subjects had been living in the USA for periods of time

ranging from a minimum of six months to a maximum of 35 years, and therefore gastro-intestinal flora may have changed, which may have influenced the levels of anti-pig and/or anti-Gal and/or anti-nonGal antibody, and (iii) the geographic regions we defined were arbitrary, and therefore may not have detected significant differences in antibody levels.

Nevertheless, with the important proviso that this study needs to be repeated in subjects who remain in the same geographic locations in which they were raised, we conclude that geographic location during childhood (and possibly other local factors at that time) may influence anti-pig, anti-Gal, or anti-nonGal antibody levels. Whether these differences are sufficient to influence the outcome of a clinical trial of xenotransplantation remains uncertain, but doubtful. The availability of GTKO pigs renders increased levels of anti-Gal antibody irrelevant, but increased levels of anti-nonGal antibody may prove problematic. The results of clinical trials of xenotransplantation may also be influenced by factors such as the age, blood group, and vaccination history of the subjects.

Methods

Sources of human sera

Under University of Pittsburgh Institutional Review Board approval, blood was drawn from 75 human volunteers of all ABO blood groups (A, B, AB, O) who had lived at least the initial 18 years of their lives in a specific region of the world (Table 1). Serum samples were collected from 51 males and 24 females. None of the volunteers had been exposed to pig antigens (except in the diet). Demographic details, including age, dietary and vaccination histories were recorded. Sera were prepared (heat-inactivated at 56°C for 30min) and stored at -80°C until use.

Pig peripheral blood mononuclear cells (pPBMC)

To detect the presence of anti-pig (anti-Gal+anti-nonGal) and anti-nonGal antibodies in the serum, PBMC were isolated from one wild-type (WT) pig and one $\alpha 1$, 3-galactosyltransferase gene-knockout (GTKO) pig (40) (Revivicor, Blacksburg, VA), which were of non-A (O) blood type, as previously described (41). Only one WT and one GTKO pig were used in all experiments to reduce variability.

Binding of human serum IgM and IgG to pPBMC by flow cytometry

Binding of human xenoreactive antibodies to pig cells was measured by flow cytometry using relative mean fluorescence intensity (rMFI), as previously described (9). All assays were repeated three times using the same WT and GKTO pPBMCs. The optimum serum concentration was determined by a preliminary study (data not shown).

Anti-Gal ELISA

Anti-Gal antibodies in serum samples were detected by anti-Gal ELISA for IgM and IgG, as previously described (42).

Statistical analyses

The statistical significance of differences was determined by Student's t, independent-samples t, or nonparametric test, one-way ANOVA, a Pearson's correlation coefficient, as appropriate, using SPSS17 (SPSS, Chicago, IL). Values are presented as mean $\pm$ SEM. Differences were considered to be significant at $p < 0.05$. When ANOVA was employed to look for differences between groups, the p value was not significant. Hence, all p values are based on the independent 't' test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The study was planned and initiated by GK and DKCC. The laboratory assays were conducted by GK, JF, HZ, MF, BE, CL, ED and HH. Pig cells were provided by DA. Statistical analyses were conducted by QS, GK and VS. The manuscript was prepared by GK, VS and DKCC and revised and approved by all authors.

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Abbreviations

Gal	Gal α 1, 3Gal
GTKO	α 1, 3-galactosyltransferase gene-knockout
PBMC	peripheral blood mononuclear cells
rMFI	relative mean fluorescence intensity
WT	wild-type

Figure 1. Human IgM and IgG binding to WT and GTKO pig PBMC (n=75)

One-sample t-test was used to compare the relative (r) MFI in IgM and IgG binding between WT and GTKO pPBMC. There were significant differences in both IgM and IgG binding between WT and GTKO pPBMC. (IgM mean rMFI 5.27 vs 1.56; $p < 0.001$; IgG mean rMFI 1.97 vs 1.31; $p < 0.001$).

Figure 2. Relationship between IgM and IgG antibody levels

(A) There was a statistically positive correlation between IgM and IgG binding to GTKO pBMC ($p < 0.001$), but not to WT pBMC ($p = 0.58$) (Pearson correlation coefficient.) (B) There was no correlation between the levels of anti-Gal IgM and IgG as measured by ELISA

Figure 3. Anti-pig IgM (A) and IgG (B) antibody levels in sera from subjects from different geographic regions

Subjects from Japan had a higher mean anti-pig IgM than those from Europe, North and South America, and South East Asia. Subjects from South Asia had a higher mean anti-pig IgM than those from Europe and South East Asia. Mean anti-nonGal IgG in subjects from Middle-Eastern countries was significantly higher than in subjects from all other geographic regions ($p < 0.05$) except Japan. Mean anti-Gal IgG was also higher in the Middle-Eastern group than in all other groups ($p < 0.01$) except South East Asia. There were no other significant differences.

Table 1

Regions and countries of origin of subjects enrolled in study

Geographic area	Countries included	Number of subjects (n)	Average Age	Gender (M/F)
Europe	Netherlands, Spain, United Kingdom	15	36	8/7
Japan	Japan	14	39	13/1
Middle-East	Egypt, Jordan, Lebanon, Turkey	8	43	8/0
North America	Canada, USA	10	27	6/4
South America	Brazil, Chile, Colombia, Cuba, Mexico	12	40	3/9
South Asia	India, Pakistan	7	29	6/1
South-East Asia	China, Korea, Malaysia, Taiwan	9	39	7/2
Total		75	36	51/24

Table 2
Mean anti-pig antibody levels in relation to geographic origin of subject

	Europe	Japan	Middle-East	North America	South America	South Asia	SouthEast Asia
number (n)	15	14	8	10	12	7	9
Anti-pig IgM*	4.26±0.42	6.67±1.22^(a)	4.72±0.73	5.88±0.90	4.50±0.39	6.80±0.68^(b)	3.45±0.78
Anti-pig IgG*	1.46±0.08	2.16±0.24	2.42±0.75	2.22±0.31	2.22±0.44	1.53±0.14	1.53±0.27
Anti-nonGal IgM*	1.44±0.09	1.60±0.12	1.95±0.33	1.36±0.13	1.85±0.25	1.46±0.22	1.38±0.24
Anti-nonGal IgG*	1.12±0.11	1.62±0.24	2.21±0.78^(c)	1.09±0.12	0.99±0.05	0.96±0.03	1.22±0.12
Anti-Gal IgM**	0.41±0.06	0.49±0.06	0.37±0.08	0.59±0.08	0.43±0.07	0.49±0.04	0.38±0.06
Anti-Gal IgG**	0.61±0.08	0.71±0.09	1.18±0.08^(d)	0.68±0.11	0.63±0.11	0.75±0.14	0.98±0.16

* Flow cytometry data shown in terms of mean rMFI ± SEM

** ELISA data shown in terms of mean OD ± SEM

^(a) For anti-pig IgM, the mean rMFI of Japan differs from Europe, South America, and South East Asia (P<0.05).

^(b) For anti-pig IgM, the mean rMFI of South Asia differs from Europe and South East Asia (P<0.05).

^(c) For anti-nonGal IgG, the mean rMFI of the Middle East differs from Europe, North and South America, and from South Asia and South East Asia (P<0.05).

^(d) For anti-Gal IgG, the mean OD of the Middle East differs from Europe, Japan, North and South America, and South Asia (P<0.01).