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Anti-Gal and Anti-Non Gal Antibody Barriers in Xenotransplantation

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1. Introduction

The many studies on the immune mechanisms contributing to xenograft rejection have identified two types of antibodies (Abs) that form barriers to transplantation of xenografts into humans: 1. Natural and induced anti-Gal Abs, and 2. Induced anti-non gal Abs. The formidable barrier of anti-Gal Abs seems to have been removed by the generation of α1,3galactosyltransferase (α1,3GT) knockout (KO) pigs. However, the second immune barrier of anti-non gal Abs may be even more formidable than that of anti-Gal Abs. The anti-non gal Ab barrier was not fully appreciated in the 1990's when much of the research was focused on overcoming the first barrier of anti-Gal mediated hyperacute rejection of pig xenografts. The anti-non gal Ab barrier still presents a challenge that requires the development of novel immunological treatments which prevent the production of these Abs. It is possible that without overcoming the anti-non gal Ab barrier it may be difficult to progress in clinical xenotransplantation beyond the use of short term bridge xenografts. Both the anti-Gal and anti-non gal barriers have been the focus of many studies in nonprimate mammal and in monkeys. This chapter does not intend to review the vast literature on anti-Gal and anti-non gal immune response in experimental animal models, but aims primarily to describe the information gained in studying anti-Gal and anti-non gal Ab response in humans. Although xenotransplantation is rarely performed in humans, I have had the opportunity of collaborating with several groups that introduced xenogeneic cells or tissues expressing α-gal epitopes into humans and study anti-Gal and anti-non gal Ab response in the sera of such patients. I believe that the information gained in these studies may contribute to the understanding of the immune response to α-gal epitopes and to xenoantigens that induce the anti-non gal Ab response in humans.

2. The anti-Gal Ab and the α-gal epitope

Anti-Gal is the most abundant Ab in humans, comprising ~1% of circulating immunoglobulins (Galili et al., 1984). This Ab is present in the serum as IgG, IgM and IgA isotypes and in various body secretions as IgG and IgA (Galili et al., 1984; Hamadeh et al., 1995; Yu et al., 1999). In recent studies, anti-Gal was found in some individuals also as an IgE Ab that can mediate a systemic allergic reaction following the infusion of the monoclonal Ab cetuximab which carries α-gal epitopes on its Fab (Chung et al. 2008). The

isotype switch into anti-Gal IgE was reported to be associated with biting of the tick *Amblyomma americanum* which transmits lime disease (Commins et al., 2011). Although anti-Gal is present in large amounts in humans it interacts with a very high specificity with a carbohydrate antigen (Ag) called the α-gal epitope (Galα1-3Galβ1-4GlcNAc-R) on glycolipids and glycoproteins (Galili et al., 1985; 1987a). Anti-Gal is produced in humans throughout life as a result of continuous antigenic stimulation by gastrointestinal bacteria with cell wall carbohydrate Ags that have a structure similar to the α-gal epitope (Galili et al. 1988a). Anti-blood group A and B Abs are also produced as a result of antigenic stimulation by the gastrointestinal flora (Springer & Horton, 1969). However, anti-Gal differs from these blood group Abs in that it is produced in all humans who are not severely immunocompromized. In individuals with blood type A and O, >80% of anti-blood group B activity is in fact by anti-Gal Abs that are capable of binding to α-gal epitopes despite of the branching fucose, as in blood group B Ag (i.e. Galα1-3(Fucα1-2)Galβ1-4GlcNAc-R) (Galili et al., 1987a; McMorrow et al., 1997). However, in blood group B and AB individuals, anti-Gal exclusively interacts with the α-gal epitope and not with other carbohydrate structures.

In contrast to protein Ags, carbohydrate Ags (with the exception of sialic acid) have no electrostatic charges. Therefore the affinity of anti-Gal to the α-gal epitope is much lower than that of anti-protein Abs. Affinity analysis performed by equilibrium dialysis using free α-gal epitope as the radiolabeled trisaccharide [^{3}H]Galα1-3Galβ1-4GlcNAc have indicated that the affinity is highly variable in different individuals and it ranges between $2x10^5$ to $6x10^6 M^{-1}$ (Galili & Matta, 1996). However, since anti-Gal is produced in very large amounts, it is very effective in inducing destruction of pig cells and tissues expressing α-gal epitopes on their surface (Galili, 1993; Good et al., 1992; Sandrin et al., 1993; Collins et al., 1994).

The proportion of B cells capable of producing anti-Gal is ~1% of circulating B cells, whereas the proportion of B cells capable of producing anti-blood group A or B Abs is 4-5 fold lower (Galili et al., 1992). This could be determined by immortalization of human blood B cells by Epstein Barr virus and the growth of such cells as individual clones. One in 100 B cells produces anti-Gal *in vitro* whereas only one in 400-500 B cell clones produces anti-blood group A or B Abs (Galili et al., 1993). Most of B cells capable of producing the anti-Gal Ab (designated anti-Gal B cells) are quiescent and only those along the gastrointestinal tract continuously produce this natural Ab. Analysis of the immunoglobulin genes in anti-Gal B cells indicated that this is a polyclonal population, however, the immunoglobulin heavy chain genes in most clones, cluster in the VH3 family (Wang et al., 1995).

3. Distribution of the α-gal epitope and anti-Gal Ab in mammals

The α-gal epitope is unique to mammals, where it is found as $1x10^6$-$30x10^6$ epitopes/cell and is completely absent in fish, amphibians, reptiles, or birds (Galili et al., 1987b, 1988b). Among mammals, α-gal epitopes are present on cells of marsupials such as kangaroo and opossum and on cells of non-primate placental mammals like mouse, rat, rabbit, bat, pig, cow, horse, cat, dog, and dolphin (Galili et al. 1987b, 1988b). The α-gal epitope is also found in similar abundance on cells of prosimians (e.g., lemurs), and New World monkeys (i.e., monkeys of South America), but not on cells of Old World monkeys (monkeys of Asia and Africa), apes (e.g., chimpanzee, gorilla and orangutan), and humans (Galili et al., 1987b, 1988b). In contrast, humans, apes and Old World monkeys are not immunotolerant to the α-gal epitope and they all produce large amounts of the natural anti-Gal Ab against it (Galili et al., 1987b).

The unique distribution of α-gal epitopes and the anti-Gal Ab in mammals is the result of the differential activity of the glycosylation enzyme α1,3galactosyltransferase (α1,3GT) which is active in the trans-Golgi compartment where it transfers galactose from the sugar donor UDP-Gal to N-acetyllactosamine (Galβ1-4GlcNAc-R) on carbohydrate chains of glycoproteins and glycolipids to synthesize the α-gal epitope. The α1,3GT gene (also referred to as *Ggta1*) is expressed in mammalian cells but is inactive in humans, apes and Old World monkeys (Galili et al. 1988b; Thall et al. 1991). This inactivation is primarily the result of various deletions in the *Ggta1* gene causing frame shift mutations in the open reading frame and the generation of pre-mature stop codons (Larsen et al., 1990; Joziasse et al., 1992; Galili & Swanson, 1992; Koike et al., 2002). Studies evaluating the expression of this pseudo-gene in humans by PCR have demonstrated its low transcription (Koike et al., 2002), however, since the protein molecule is truncated, it is devoid of catalytic activity. Truncation studies in the New World monkey α1,3GT have indicated that deletion of as few as three amino acids at the C-terminus is sufficient to result in complete lose of catalytic activity (Henion et al., 1994). Comparison of the sequence of this pseudogene in humans and in other primates led us to suggest that the α1,3GT gene was inactivated in ancestral Old World primates, after apes and monkeys diverged from each other, 20-25 million years ago (Galili & Swanson, 1992; Galili & Andrews, 1995).

4. The rejection of xenografts by the anti-Gal Ab

Several seminal studies demonstrated *in vitro* the destruction of cells by complement mediated cytolysis (Good et al., 1992, Sandrin et al., 1993) or by Ab dependent cell mediated cytotoxicity (ADCC) (Galili, 1993; Watier et al., 1996) due to interaction of human anti-Gal Ab with α-gal epitopes on pig cells or on monkey cells transfected with α1,3GT gene and thus expressing the α-gal epitopes on their cell membrane. In *in vivo* studies, transplantation of pig or New World monkey xenografts into Old World monkeys was found to result in *in situ* binding of anti-Gal to α-gal epitopes on endothelial cells of grafts, complement mediated lysis of these cells due to this Ag/Ab interaction, the ensuing collapse of the vascular bed and hyper acute rejection of the xenograft (Collins et al., 1994). Subsequent studies demonstrated the direct association between *in vivo* neutralization of anti-Gal by α-gal oligosaccharides and delay in hyperacute rejection (Simon et al., 1998), and the association between removal of anti-Gal by adsorption on affinity columns and delay in xenograft rejection (Kozlowski et al., 1998; Xu et al., 1998). These studies directly proved that the anti-Gal Ab is the Ab mediating hyperacute rejection of xenogafts *in vivo*.

5. Stimulation of the immune system to produce anti-Gal Ab in xenograft recipients

Anti-Gal is present in very high amounts in all individuals who are not severely immunocompromized. Nevertheless, the human immune system is capable of producing this Ab in much higher titers due to the activation of many of the quiescent anti-Gal B cells throughout the body. As indicated above, ~1% of B cells in the blood have the capacity of producing the anti-Gal Ab, but are in a quiescent state (Galili et al., 1993). However, in individuals who are transplanted with xenografts that present α-gal epitopes, these quiescent B cells are readily activated to produce the anti-Gal Ab. The activated anti-Gal B

cells further undergo isotype switch as well as affinity maturation, ultimately resulting in an increase of ~100 fold in the titer of this Ab.

Anti-Gal Ab response to α-gal epitopes on xenogeneic cells could be monitored in an ovarian carcinoma patient who received an experimental gene therapy treatment for destruction of tumor cells by ganciclovir (Galili et al., 2001). The patient received 3 intraperitoneal infusions in 7 weeks intervals, each of $6x10^9$ mouse fibroblasts that released a replication defective retro-virus containing the thymidine kinase gene. Tumor cells infected *in situ* by the virus are killed by subsequent administration of ganciclovir (Link et al., 1996). Since the infused mouse fibroblasts present multiple α-gal epitopes (Galili et al., 1988b), this treatment is immunologically similar to the transplantation of xenograft cells expressing α-gal epitopes in humans. Anti-Gal activity in the serum of the patient was studied by ELISA with synthetic α-gal epitopes linked to bovine serum albumin (α-gal BSA) as solid phase Ag. Within one week post infusion of mouse fibroblasts, the titer of anti-Gal IgG Ab increased by ~10 fold, and two weeks post post infusion by ~100 fold (Galili et al., 2001) (Fig. 1). The Ab activity remained at that high level after the second and third infusions. This extensive anti-Gal Ab response was the result of activation of the many anti-Gal B cell clones that engage α-gal epitopes on the glycoproteins released from the infused mouse fibroblasts. Studies measuring the concentration of anti-Gal in the serum (by isolation on an α-gal column) and its affinity (by dialysis of radiolabled free α-gal epitope in the form of trisaccharide [Galili & Matta, 1996]) indicated that the 10 fold increase in anti-Gal titer within the first week post transplantation was the result of an increase in the concentration of anti-Gal Ab in the serum (i.e. increased production of the Ab), whereas the additional 10 fold increase within the second week was associated with a corresponding increase in the affinity of this Ab (Galili et al., 2001). These findings strongly suggest that the increase in the titer of anti-Gal Ab observed after one week is the result of activation of quiescent anti-Gal B cells by α-gal epitopes on glycoproteins released from the xenograft cells, thereby increasing the concentration of this Ab in the serum. The subsequent increase in affinity of the Ab observed at the end of the second week is probably a result of affinity maturation by the process of somatic mutations within anti-Gal B cell clones. This process occurs after the initial activation of the quiescent anti-Gal B cells.

The increase in anti-Gal Ab response was mostly (~90%) of the IgG2 subclass and the remaining was of the IgG3 subclass. No significant increase was observed in the activity of anti-Gal IgG1, IgG4, IgM or IgA (Galili et al., 2001). This suggests that the isotype switch of anti-Gal B cells stimulated by α-gal presenting xenoglycoproteins (α-gal glycoproteins) is quite rapid from IgM to IgG2 and IgG3. It should be stressed, however, that anti-Gal IgM is naturally present in large amounts in human serum (Hamadeh et al., 1995; Yu et al., 1999) and anti-Gal IgA and IgG are present in various secretion such as saliva, milk, colostrum and bile (Hamadeh et al., 1995).

Activation of anti-Gal B cells and production of the anti-Gal Ab by plasma cells seems to occur as long as there are glycoproteins with α-gal epitopes in the body. The decrease in anti-Gal titer observed 4 and 7 weeks after the first infusion of mouse fibroblasts (Fig. 1) suggests that after the anti-Gal mediated destruction of these cells and the elimination of α-gal glycoproteins, anti-Gal B cells cease to be activated and to differentiate into plasma cells secreting the Ab. Since the period of Ab secretion by plasma cells is limited, activity of anti-Gal Ab decreases in the serum within a short period after the elimination of glycoproteins

with α-gal epitopes. It is of interest to note that anti-Gal production after the second and third infusions of mouse fibroblasts was not higher than that observed after the first infusion (Fig. 1). It is probable that high affinity anti-Gal IgG molecules produced in large amounts, effectively mask α-gal epitopes on glycoproteins released from the infused cells. Such masking limits the extent of further B cell activation and keeps that activation at a level of Ab production similar to that observed 2 weeks after the first infusion.

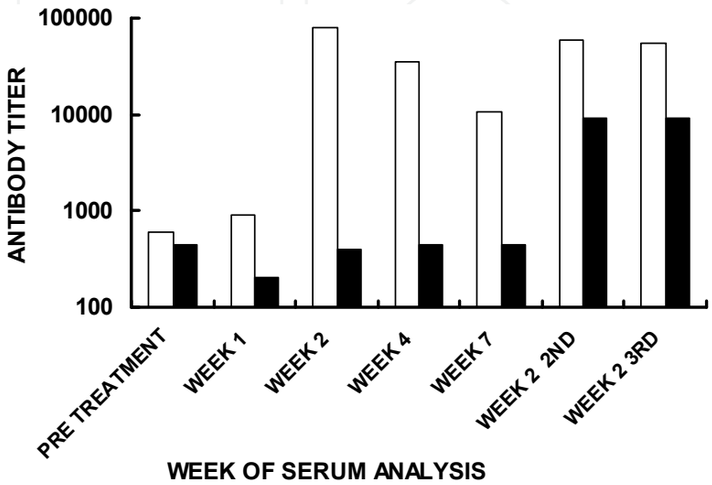

Fig. 1. Production of anti-Gal (open columns) and anti-non gal IgG Abs (closed columns) in an ovarian carcinoma recipient of $6x10^9$ mouse fibroblasts. The patient received 3 intraperitoneal infusions of the fibroblasts in 7 week intervals. Ab titers were determined prior to treatment, 1, 2, 4 and 7 weeks post treatment, 2 weeks after the second (2^{nd}) and third (3^{rd}) treatments. The titers are presented as reciprocals of serum dilution yielding half the maximum binding in ELISA. The solid phase Ags used for the study were synthetic α-gal epitopes linked to BSA (α-gal BSA) for anti-Gal Ab analysis and the mouse fibroblast cell line used in the treatment for anti-non gal Ab analysis. Sera were depleted of anti-Gal Ab prior to performing the assay for anti-non gal Abs (modified from Galili et al., 2001).

A similar extensive increase in anti-Gal titer was observed in patients with impaired liver function, who were treated by temporary extracorporeal perfusion of their blood through a pig liver (Cotterell et al., 1995; Yu et al. 1999). The increase in anti-Gal titer in these patients implies that the release of xenoglycoproteins from the pig liver, perfused for several hours, was sufficient to induce the activation of the many quiescent anti-Gal B cells for production of the anti-Gal Ab. Interestingly, a similar rapid and extensive increase in anti-Gal IgG activity was observed in cynomolgus monkeys (an Old World monkey) implanted with pig meniscus cartilage which contains an abundance of α-gal epitopes (Galili et al., 1997). This suggests that non-human primates capable of producing anti-Gal Ab also have multiple quiescent anti-Gal B cells as those in humans.

6. Anti-Gal Ab production in immunosuppressed xenograft recipients

The extensive activation of anti-Gal B cells by α-gal epitopes on xenografts is very difficult to suppress. This can be inferred from the studies on anti-Gal response in diabetic patients who were transplanted by Groth and colleagues with an allogeneic kidney together with pig

fetal islet cell clusters (Groth et al., 1994; Galili et al., 1995). These studies were the first to demonstrate the induced anti-Gal Ab response in xenograft recipients. Pig islet cell clusters were generated by culturing of fetal pig pancreatic tissues (Korsgren et al. 1988). The fetal islet cells proliferate *in vitro* and form clusters of islet cells which were transplanted at a volume corresponding to 2-6 ml packed cells (Groth et al., 1994). The islet cell clusters were implanted under the transplanted kidney capsule or infused into the portal vein in the liver. Recipients of the kidney allograft and the islet cell xenografts displayed an increase of 20-80 folds in anti-Gal titer within the period of 25-50 days post transplantation (Galili et al., 1995). As with the ovarian carcinoma patient infused intraperitoneal with the mouse fibroblasts (Galili et al., 2001), the increase in anti-Gal activity was mostly in the IgG isotype and to a much lesser extent of IgM and IgA isotypes. However, unlike the ovarian carcinoma patient, the transplanted diabetic patients were heavily immunosuppressed, to the extent that the immune system did not reject the kidney allograft (Groth et al., 1994). The increased production of anti-Gal Ab in these immunosuppressed patients suggests that currently used immunosuppressive protocols, which are effective enough to prevent allograft rejection, fail in preventing much of the activation of anti-Gal B cells by α-gal glycoproteins released from the xenograft.

Studies in α1,3galactosyltransferase knockout mice that are capable of producing anti-Gal Ab have indicated that stimulation of anti-Gal B cells by α-gal epitopes on xenografts to produce the anti-Gal Ab requires T cell help. However, the α-gal epitope by itself (like other carbohydrate chains of the complex type) can not activate helper T cells (Tanemura et al., 2000). T cell help for anti-Gal B cells is provides by helper T cells activated by the multiple xenogeneic peptides processed and presented by antigen presenting cells (Tanemura et al., 2000; Galili 2004). Interestingly, in the absence of T cell help, interaction between α-gal epitopes and B cell receptors on anti-Gal B cells results in induction of immune tolerance to α-gal epitopes and prevention of anti-Gal Ab production in the tolerized recipients (Mohiuddin et al., 2003; Ogawa et al., 2003). A similar tolerance induction was achieved by bone marrow chimerism with α-gal epitope presenting syngeneic bone marrow cells (Bracy et al., 1998).

7. Elimination of the anti-Gal Ab barrier by the use of α1,3galactosyltransferase knockout pigs

As indicated above, the natural anti-Gal Ab and the elicited anti-Gal Ab are of no clinic significance in xenograft recipients, if the xenograft is obtained from α1,3galactosyltransferase knockout pigs. These pigs have been generated by targeted disruption (knockout) of the α1,3galactosyltransferase gene (Phelps et al., 2003; Kobler-Simond et al., 2004; Yamada et al., 2005; Takahagi et al., 2005; McGregor et al., 2011). These pigs lack α-gal epitopes and their organs do not induce an anti-Gal response when transplanted into primates (Chen et al., 2005; Ezzelarab et al., 2006; Hisashi et al., 2008; Yeh et al., 2010). Thus, in contrast to rapid (hyperacute) rejection of wild type (WT) pig organs in monkeys (observed within <1h to several hours), pig organs from α1,3galactosyltransferase knockout pigs (α1,3GT KO pigs) survive in monkeys for weeks to several months prior to rejection (Yamada et al., 2005; Chen et al., 2005; Kuwaki et al., 2005; Tseng et al., 2005; Hisashi et al., 2008). In the absence of anti-Gal response the next immune obstacle in xenotransplantation became apparent- the production of anti-non gal Abs against

xenoantigens of the graft which are not α-gal epitopes. Most of these xenoantigens are multiple pig proteins that are immunogenic in humans.

8. Most of the proteins within pig xenografts are expected to be immunogenic in humans

The amino acid sequence of most homologous (orthologous) proteins varies in different mammals. There are only few highly conserved proteins, such as histones and collagen in which amino acid sequence changes have been minimal because of functional constraints. However, most genes accumulate random mutations (referred to as the evolutionary molecular clock [Wilson & Sarich, 1969]) which result in variations in amino acid sequence. Since humans and pigs (as well as other nonprimate mammals) have been evolving independently along separate lineages for an evolutionary period estimated to be ~75 million years (Pilbeam 1984), each has accumulated multiple lineage and species specific mutations. The proportion of such mutations varies in different regions of a given protein, based on functional constraints, e.g. in a membrane bound receptor there are more mutations in the tether region than the ligand binding region. Regardless of their location, mutations form immunogenic amino acid sequences in pig proteins, since they are absent in humans. The immune system can react against very small changes in various Ags. This can be inferred from the immune response to blood group Ags where the presence of one small N-acetyl group (CH_3CONH) in blood group A and its absence in blood group B is sufficient for inducing production of anti-A Abs in blood group B individuals. Because most pig proteins contain some amino acid sequences that are different from those in homologous proteins in humans, it is likely that most pig proteins are immunogenic in humans and can induce an Ab response in xenograft recipients. Therefore, humans transplanted with pig cells or organs may produce hundreds and possibly thousands of Ab specificities against pig xenogeneic peptides. These Abs have been referred to as anti-non gal Abs (Galili et al., 2001). Since there are very large numbers of undefined xenoantigens that elicit anti-non gal Ab response, pig tissues or cells may serve as antigenic preparations for analysis of such Abs. For such analysis, anti-Gal Abs have to be removed from the tested human sera prior to the assay. This anti-Gal depletion is feasible by adsorption of anti-Gal Ab on rabbit RBC or on glutaraldehyde rabbit RBC (Galili et al., 2001; Stone et al., 2007) since these RBC present the highest number of α-gal epitopes among mammalian RBC (Ogawa & Galili, 2006). As described below, studies on anti-non gal Abs in xenograft recipients have indicated that their production is very different from that of elicited anti-Gal Abs.

9. Anti-non gal Abs in a recipient of mouse fibroblasts

The studies on the Ab response in the ovarian carcinoma patient receiving intraperitoneal infusion of mouse fibroblasts led to the first report on production of anti-non gal Abs in humans that are recipients of a xenograft (Galili et al. 2001). Although, in this patient the anti-non gal Ab response was against mouse proteins, the results of this analysis are also applicable to the understanding of anti-non gal immune response to pig proteins. This is since the evolutionary distance between humans and rodents does not differ significantly from the distance between humans and pigs (i.e. ~75 million years of evolution in separate lineages since the "great mammalian radiation") [Pilbeam 1984]. Anti-non gal Ab activity in that patient could be determined by ELISA with the mouse fibroblast cell line used in the

treatment, as a solid phase Ag. These fibroblasts strongly adhere to ELISA wells following the overnight drying of the cell suspension in the wells. Serum samples from various time points post intraperitoneal infusion were the same as those used for anti-Gal analysis (Fig. 1), however, the sera were depleted of anti-Gal Abs by adsorption on rabbit RBC (50%) on ice (Galili et al., 2001).

Although anti-Gal IgG Ab activity increased by ~14 days post administration of the mouse fibroblasts, no induced anti-non gal Ab production was detected at that time point (Fig. 1). These Abs were not detected even 7 weeks after the first infusion. However, within 2 weeks after the second infusion, a robust anti-non gal Ab response was observed in the serum of the patient (Fig. 1). Western blot analysis indicated that the Abs produced bound to multiple proteins in the mouse fibroblasts, confirming the multiclonality of the B cell response (Galili et al. 2001). It is probable that there are also many T cell clones that are activated by xenogeneic peptides processed and presented by the antigen presenting cells of the treated patient. Nevertheless, the lack of detectable induced anti-non gal Ab response after the first fibroblast infusion strongly supports the assumption that the initial number of anti-non gal B cells in each of the multiple B cell clones reacting against xenoantigens is very low. By the time B cells in these multiple clones proliferate to the extent required for producing detectable levels of anti-non gal Abs, the stimulatory fibroblasts have disappeared due to anti-Gal Ab mediated destruction. Thus, production of anti-non gal Abs is detectable only after the second infusion of mouse fibroblasts which provides an antigenic boost for the activation of anti-non gal memory B cells. As shown below, anti-non gal Abs appear at earlier time point in recipients of pig tissue (tendon) because of the continuous antigenic stimulation by the pig xenoantigens. The subclass distribution of the induced anti-non gal Abs was found to be IgG1>IgG2>IgG3>IgG4 (Galili et al. 2001).

It is of interest to note that the titer of anti-non gal Abs measured after the third infusion (performed 7 weeks post second infusion) did not differ from that after the second infusion (Fig. 1). These observations strongly suggests that, as with anti-Gal Ab response, anti-non gal Ab response is subjected to a self limiting dynamic regulatory mechanism. This self limiting production of anti-non gal Abs may be mediated by such Ab molecules that bind to the immunogenic peptide epitopes and mask them, thereby preventing additional stimulation of the corresponding B cells.

10. Anti-non gal Ab response in recipients of pig ligament

A phase I clinical trial on replacement of torn anterior cruciate ligament (ACL) with pig patellar tendon provided a unique opportunity for monitoring anti-non gal Ab response in the absence of immunosuppression in humans, for a period of 2 years (Stone et al. 2007). The implanted ligaments and the two attached bone blocks were treated with recombinant α-galactosidase in order to eliminate α-gal epitopes. This enzymatic treatment was performed in order to attenuate the immune response to the implant by preventing the induction of anti-Gal Ab response which can be detrimental to the implant. In addition, the ligaments underwent mild cross-linking by incubation for 12 hours with 0.1% glutaraldehyde. It was assumed that in the absence of anti-Gal response, destruction of the pig ligament mediated by anti-non gal Abs will be slowed due to the cross-linking. The slowed destruction of the cross-linked ligament will enable concomitant regeneration of the ligament tissue (ligamentization) by infiltrating fibroblasts which align with the pig collagen fiber scaffold and produce new

collagen fibers. It was further assumed that the similarity in the rates of pig ligament destruction and ligamentization by human fibroblasts will maintain the biomechanical characteristics of the implanted ligament while it is gradually replaced by the human tissue. Five patients receiving such implants 9-10 years ago continue to display normal joint activity.

Anti-non gal Ab response was studied in implanted patients by ELISA with pig ligament homogenate as solid phase Ag, using sera that were depleted of anti-Gal Ab. Induction of anti-non gal Ab production was determined by comparison of the post-implantation Ab activity at various serum dilutions with that in the pre-implantation base-line activity. The induced anti-non gal Ab response peaked 2 - 6 months post implantation (Fig. 2) (Stone et al. 2007). This Ab response was detectable also after 1 year, but it returned to the pre-implantation level after 2 years. These observations suggest that as long as the pig tissue is present within the recipient, the immune system is stimulated to produce anti-non gal Abs. However, due to the gradual replacement with human ligament tissue, the amount of pig ligament tissue is decreasing, so that by 12 months anti-non gal Ab response is lower than in the peak of 2-6 months. By 24 months, the pig ligament seems to be completely replaced by human ligament tissue therefore there is no antigenic stimulation for the production of anti-non gal Abs.

Anti-non gal IgG Ab activity in the pig ligament recipients increased on average by 5-10 folds in comparison with pre-implantation serum in each of the patients (Stone et al., 2007). This increase is much lower than the ~100 fold increase in anti-Gal Ab activity observed in the patient receiving intraperitoneal infusion of mouse fibroblasts. The difference is likely to be due to the much higher number of quiescent anti-Gal B cells (~1% of B cells [Galili et al., 1993]) which are rapidly activated by α-gal epitopes on xenoglycoproteins. The ultimate number of anti-non gal B cells at the peak of the immune response is likely to be much lower in each of the individual clones, thus, the overall immune response is significantly lower than that of the anti-Gal response.

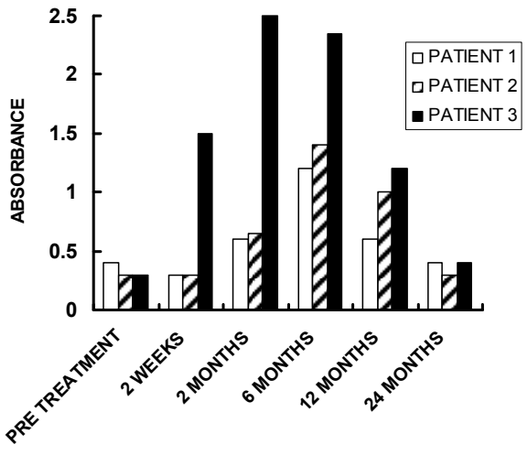

Fig. 2. Anti-non gal Ab (IgG) activity in the sera of 3 patients implanted with pig patellar tendon for replacement of torn ACL. The pig tendons were treated with α-galactosidase for destruction of α-gal epitopes and cross-linked mildly with glutaraldehyde prior to implantation. Ab activity was determined by ELISA with pig ligament homogenate as solid phase Ag. Sera were depleted of anti-Gal Ab prior to analysis. Ab binding was determined at serum dilution of 1:640 and presented as O.D. (optical density units) at the various time points (modified from Stone et al., 2007).

The specificity of the anti-non gal Abs could be studied by Western blots. Pre-implantation sera depleted of anti-Gal Abs displayed no Ab binding to pig ligament proteins or to pig kidney proteins. However, sera obtained 6 months post implantation contained anti-non gal Abs that bound to multiple pig ligament proteins. This large number of Ab specificities was indicated by the immunostaining of the blot as a smear rather than as individual bands (Stone et al., 2007). Many of stained proteins were also found in pig kidney preparations, implying that some of the proteins inducing anti-non gal Ab response are not specific to the ligament and are present in other tissues, as well. Blots of human ligament proteins were also studied for binding of anti-non gal IgG Abs. Despite the extensive binding of Abs to pig ligament proteins, no binding was observed with human ligament proteins. This strongly suggests that exposure of the human immune system to pig proteins and the extensive production of Abs against such proteins does not result in breakdown of immune tolerance to self Ags and no auto-Abs are generated.

It is not clear as yet whether the anti-non gal Ab response also includes Abs to carbohydrate Ags other than the α-gal epitope. A number of studies demonstrated the production of natural Abs to N-glycolylneuraminic acid in humans and the presence this epitope on pig cells (Zhu & Hurst, 2002; Miwa et al., 2004; Taylor et al., 2010). However, analysis of sera from monkeys sensitized to α1,3GT KO pig tissue demonstrated no significant elevation in the activity of such Abs (Yeh et al., 2010).

11. Anti-non gal Ab response in immunosuppressed recipients

The study of sera in diabetic recipients of pig fetal islet cell clusters enabled the assessment of anti-non gal Ab production in recipients of pig fetal islet cell xenograft and of a kidney allograft. These recipients were immunosuppressed to the extent that they did not reject the allograft (Groth et al., 1994). As indicated above, the immunosuppressive treatment in these patients did not prevent the induced anti-Gal immune response against α-gal epitopes on xenoglycoproteins. In order to estimate the proportion of anti-Gal and anti-non gal Abs of the total Ab binding to pig cells, binding of IgG Abs to pig PK15 kidney cells was measured in serum of transplanted patients and compared to the binding of Abs after anti-Gal depletion by binding of the Ab to α-gal epitopes linked to silica beads. Ab binding to pig cells in serum depleted of anti-Gal was 6-25% of the total IgG binding (Galili et al., 1995). These findings suggest that anti-Gal response comprised the majority of the human immune response to pig xenograft. Nevertheless, a significant proportion of the Abs was anti-non gal Abs produced despite immunosuppression effective enough to prevent the rejection of the kidney allograft.

Anti-non gal Ab production in the immunosuppressed recipients of kidney allograft and pig islet cell xenografts raises the question of whether these Abs are potent enough to mediate rejection of the xenograft. There is no direct information to address this question. Studies of xenotransplantation under immunosuppression of wild type pig heart and kidney into monkeys demonstrated anti-non gal Ab production, even if the xenograft was rejected within few days (Buhler et al., 2003; Lam et al., 2004; Chen et al., 2005; Ezzelarab et al., 2006). Transplantation of α1,3GT KO pig heart or kidney in monkeys subjected to a variety of immunosuppressive protocols resulted in survival of the xenografts for much longer periods (from several days up to 3 months [even up to 6 months in one recipient of heart xenograft]) than survival of xenografts from wild type pigs presenting multiple α-gal epitopes (Kuwaki et al., 2005; Chen et al., 2005; Tseng et al., 2005; Chen et al., 2006; Ezzelarab et al., 2006; Hisashi et

al., 2008). Ultimately, all xenografts were rejected and the recipient monkeys were found to produce anti-non gal Abs which could be detected *in vitro* as Abs binding to α1,3GT KO pig cells. Although not directly proven, it is probable that these anti-non gal Abs contribute significantly to the observed rejection of α1,3GT KO pig xenografts.

12. Challenges in preventing anti-non gal Ab response in xenograft recipients

The prevention of anti-non gal Ab response in a xenograft recipient is a formidable challenge. As discussed above, this Ab response is the result of activation of multiple B cell clones against a very large number of immunogenic peptides on many pig proteins. It is probable that the multiple immunogenic peptides processed and presented by antigen presenting cells activate a very large number of helper T cell clones that facilitate anti-non gal Ab response. The studies mentioned above, which have demonstrated anti-non gal Ab response in immunosuppressed pig xenograft recipient monkeys imply that the immunosuppression protocols presently used for preventing allograft rejection do not completely prevent anti-non gal Ab response. Thus, a major challenge in xenotransplantation is the development of immunosuppressive drugs and protocols that prevent anti-non gal immune response but do not completely eliminate the ability of the immune system to protect against microbial pathogens. It is not clear at present whether such a selective immune suppression against multiple xenoantigens but not against microbial Ags is feasible. An alternative approach for preventing anti-non gal Ab response may be the induction of immune tolerance to the multiple pig xenoantigens. One method studied for inducing such a tolerance in kidney xenograft recipients has been the thymo-kidney xenograft of α1,3GT KO pigs (Yamada et al., 2005). Pig thymus tissue is placed under the pig kidney capsule several weeks prior to transplantation in order to achieve vascularization of this tissue. The thymus component of the xenograft is expected to tolerize the recipient against pig xenoantigens (Yamada et al., 2005). Pig thymo-kidney xenografts that were transplanted into monkey recipients survived for almost 3 months, a much longer period than the survival period of kidney xenografts lacking the thymus component (Yamada et al., 2005; Griesemer et al., 2009).

It may be possible that xenograft recipients will ultimately have to be tolerized to pig xenoantigens by manipulating their immune system prior to the xenotransplantation procedure. One theoretical possibility may be the effective pre-transplantation elimination of B cells of the patients. Newly emerging B cell clones with anti-pig Ag specificity that develop in the presence of the xenograft Ags (i.e. post transplantation) may be deleted from the repertoire at the differentiation stage of immature B cells in which B cells engaging Ags are eliminated or undergo receptor editing (Sandel & Monroe 1999; Nemazee et al., 2000). It is not known at present whether elimination of these B cells will suffice for preventing rejection, or is T cell elimination required as well. An additional tolerance induction method that is being explored is the administration of pig bone marrow cells in order to induce bone marrow chimerism which may prevent an immune response to pig xenoantigens. α1,3GT KO pig bone marrow cells were reported to survive in two out of four monkeys for at least 4 weeks (Griesemer et al., 2010). It is not known as yet whether such chimerism can be maintained for much longer periods and if it can prevent anti-non gal Ab response. In addition, it is not clear whether such chimerism can tolerize against xenoantigens that are not present on bone marrow cells (e.g. Ags specific to the pig xenograft organ). All these considerations imply that although the anti-Gal barrier has been effectively overcome, much

research is still required for developing methods to overcome the anti-non gal immune response against the wide range of pig xenoantigens that are immunogenic in humans.

13. Concluding remarks

Two types of Abs form immune barriers in xenotransplantation: anti-Gal and anti-non gal Abs. The anti-Gal Ab is naturally present in humans in large amounts. The titer of anti-Gal Ab further increases in xenograft recipients by 30-100 fold because of the rapid activation of quiescent anti-Gal B cells which comprise ~1% of circulating B cells in humans. Production of anti-non gal Abs in xenograft recipients is the result of the immune response against the multiple pig proteins that are immunogenic in humans because of mutations in orthologous pig proteins. Anti-non gal Abs are produced by a large number of B cell clones with Ab specificity to the many pig peptide sequences that are not present in humans. Both anti-Gal and anti-non gal Abs are produced in humans despite immune suppression that is effective enough in preventing allograft rejection. Anti-Gal and anti-non gal Abs are continuously produced as long as the corresponding Ags are present in the treated patient. The generation of α1,3galactosyltransferase knockout pigs enabled the elimination of anti-Gal barrier in xenotransplantation since these pigs lack α-gal epitopes. However, overcoming the anti-non gal Ab barrier requires the development of novel methods that selectively prevent the induced production of these Abs while maintaining protective immune response against various pathogens.

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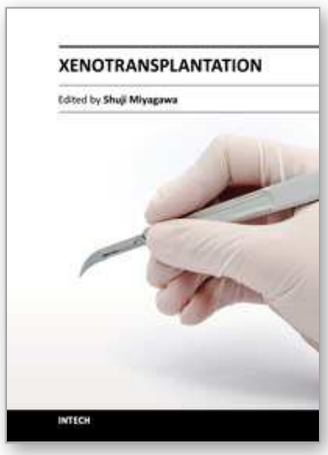

Xenotransplantation

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Accompanied by the advent of animal cloning, the technique of nuclear transfer produced alpha1,3-galactosyltransferase-knockout (Gal-KO) pigs in many institutes, including the ones in Japan, at the beginning of 21st Century. In addition, the controversy of the risks of PERV has gradually minimized, because of the fact that there are no cases of PERV infections reported in humans. Furthermore, a large clinical wave for islet allotransplantation resumed the interest of xenotransplantation, especially porcine islet transplantation and some exceptions. Clinical trials were done in many countries so far, such as Sweden, China, Mexico, USA (Inventory of Human Xenotransplantation Practices - IXA and HUG in collaboration with WHO). In addition, a new clinical trial was approved by the government, and resumed the porcine islet transplantation research in New Zealand two years ago.

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