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## LOW ANTI-PIG ANTIBODY LEVELS ARE KEY TO THE SUCCESS OF SOLID ORGAN XENOTRANSPLANTATION – BUT IS THIS SUFFICIENT?

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### Keywords

Antibodies; anti-pig; Xenotransplantation

### Background

Research in the field of pig-to-primate xenotransplantation has made significant advances during the past two years, with life-supporting pig kidneys in nonhuman primates (NHPs) surviving for many months or even more than a year (1–3, and Tector AJ, personal communication). The results have been so encouraging that clinical trials are in the early stages of planning (4,5). There are some data that suggest that genetically-engineered pig grafts transplanted into NHPs selected for no or low levels of anti-pig antibody, particularly IgG, survive longer than grafts transplanted into NHPs with higher levels of antibody.

In prospective clinical trials, if the initial patients are selected on the basis of no or low detectable levels of anti-pig antibody, will this be sufficient to ensure consistent prolonged graft survival or will additional genetic modifications, e.g., expression of human complement- or coagulation-regulatory proteins, be essential? Data from selected studies in which anti-pig antibody levels were known are here reviewed.

Evidence that selection of NHPs with no or low levels of anti-pig antibodies is associated with prolonged pig organ graft survival has slowly accumulated since the first studies of organ transplantation from pigs that did not express the important galactose- $\alpha$ 1,3-galactose (Gal) antigens were reported in 2005 (6–8). The baboon recipients were selected for low anti-nonGal antibody levels, i.e., antibodies directed to the remaining pig antigens that were not Gal (nonGal antigens) (9,10) (Table 1). Survival of heterotopic (non-life-supporting) heart grafts extended to 6 months (6,7) and of life-supporting kidney grafts to a little less than 3 months (8). The discrepancy in graft survival between heart and kidney may not be

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simply related to whether or not the organ was life-supporting, as molecular differences may contribute to the poorer outcome of pig kidney grafts in NHPs (11). However, whether orthotopic (life-supporting) heart xenograft survival will match heterotopic (non-life-supporting) heart xenograft survival has yet to be determined.

In baboons that did not develop complications associated with the immunosuppressive regimen, e.g., infection, all heterotopic heart grafts developed thrombotic microangiopathy (Figure 1) (12) and the recipient developed features of a consumptive coagulopathy (though this has not been reported in all studies [discussed in 13]). This was attributed to vascular endothelial activation by a low level of anti-pig antibody combined with incompatibility of coagulation factors between pig and baboon. As there was no evidence for an elicited (T cell-mediated) antibody response, this activation was presumably associated with a continuing low level of production of natural (preformed) antibody, possibly in association with such factors as complement deposition and innate immune cell activity. These encouraging results could not be repeated when the selection of the recipient baboons was *not* based on low antibody levels (Table 1) (14,15).

The hypothesis that low antibody levels are important gained considerable support from studies in the GTKO/CD55 pig-to-rhesus monkey kidney transplantation model (1). Using an identical immunosuppressive regimen (based on costimulation blockade), a kidney transplanted into a monkey with a high level of anti-nonGal antibody functioned for only 6 days, whereas two with low antibody levels functioned for 6 and 10 months, respectively (Table 1). However, grafts were eventually lost through delayed antibody-mediated rejection and coagulation dysfunction (Adams A, personal communication), again suggesting that very gradual antibody binding to the graft vascular endothelium (combined with coagulation incompatibilities between pig and NHP) may have been detrimental.

A low level of antibody (or even no detectable antibody) may therefore be insufficient to ensure truly long-term graft survival, and may not even be sufficient for short-term survival. This is exemplified by a recent study in four baboons (all selected for low antibody levels, and all treated with an identical experimental protocol), in which the expression of different specific human complement- and coagulation-regulatory proteins on the pig kidney grafts resulted in a remarkable difference in graft survival from 12 days (n=2) to >7 months (n=2) (Table 1) (3). (The poor results when kidneys from GTKO/CD46/hThrombomodulin pigs were transplanted, which were in contrast to excellent results when identical hearts were transplanted (16), is likely related to the much greater expression of hThrombomodulin in the heart than in the kidneys in these pigs [Ayares D, personal communication].) Expression of an effective human coagulation-regulatory protein was therefore essential in obtaining prolonged graft survival.

Furthermore, the expression of human complement- +/- coagulation-regulatory proteins can result in relatively prolonged graft survival even when the recipient NHPs have *not* been selected for low antibody levels (Table 1) (2,17,18), with survival extending to >2 years (16).

These observations supported earlier studies in which the expression of a human complement-regulatory protein on GTKO pigs extended early graft survival in non-selected

baboons (19,20). It should also be remembered that the original studies of pig organ transplantation in NHPs carried out by White, Cozzi, and their colleagues, used pigs in which *no* pig antigens had been deleted (i.e., wild-type pigs), but which expressed a human complement-regulatory protein, CD55 (21). When the pig was transgenic for a human complement-regulatory protein, extensive studies in NHPs indicated that heart and kidney graft survival was significantly extended when compared with that of wild-type pig organs despite the fact that no attempt was made to reduce the levels of anti-pig antibody (reviewed in 22 and 23).

## Recent progress

In the early pig-to-NHP organ transplantation studies, the only known pig antigen against which humans and NHPs had natural antibodies was Gal (24), and so it was only possible to delete expression of this single antigen. It has long been known that pigs (but *not* humans) express N-glycolylneuraminic acid (Neu5Gc) (25), and therefore humans make anti-Neu5Gc antibodies (26). This is irrelevant to studies in NHPs as all Old World NHPs express Neu5Gc, as do pigs (reviewed in 27). Human serum IgM and IgG binding to cells from 'double-KO' pigs, i.e., pigs that express neither Gal nor Neu5Gc (GTKO/CMAHKO pigs), is significantly less than to cells from GTKO pigs (Figure 2) (28).

More recently, a third pig antigen target has been identified, Sd<sup>a</sup>, the product of  $\beta$ 1,4N-acetylgalactosaminyltransferase-2 ( $\beta$ 4GalNT2) (29). When using target cells from pigs in which expression of all three antigens had been deleted ('triple-KO' [TKO] pigs) (30,31), a relatively large number of humans (approximately one-third of the subjects tested) were identified who appeared to have *no* antibody binding to these cells (32). It can be argued, therefore, that such patients would do well after receiving a graft from a TKO pig, just as patients with no pre-existing donor-specific allo-antibodies do well after organ allotransplantation (as long as an elicited antibody response is prevented).

Preventing a donor-specific antibody response, however, has proved difficult even following allotransplantation. Many patients undergoing kidney allotransplantation, who had no detectable donor-specific antibodies at the time of the transplant, develop *de novo* antibodies after transplantation. It seems just as likely, or even more so, that such *de novo* antibodies will develop against a pig xenograft.

Several studies suggest that there is no cross-reactivity between those antibodies directed to human MHC (anti-HLA antibodies) and those directed to swine MHC (anti-SLA antibodies) (33–36), though others indicate that this is not always so (32, and reviewed in 33). Indeed, Oostingh et al (37) provided data to suggest that almost half of patients with a panel reactive antibody level of >64% had antibodies that cross-reacted with SLA. Although the data reported by Oostingh et al have not been confirmed by several other groups, donor MHC may represent a problem with regard to both pre-existing and *de novo* antibodies.

In those humans in whom a low level of anti-TKO pig antibodies was identified, there was evidence that some of these antibodies may be directed to MHC swine leukocyte class I antigens (SLA-I) (32). The discrepancies in the results from different studies may be related

to (i) the SLA phenotype of the specific pig from which the cells were derived, and/or (ii) the number of human sera tested, which was far greater in the studies by Martens and his colleagues (32). Genetic engineering techniques will likely allow the specific SLA targets to be deleted in these pigs, allowing the patient to receive a pig graft against which he/she has *no* antibodies.

In addition to the above (and possibly other) protein-directed antibodies, some human anti-TKO pig antibodies may be directed to hitherto unidentified pig glycans, although the level of these antibodies is low. When these antigen targets are identified, this will allow their knockout but, in view of the very low binding of antibodies in most humans to TKO pig cells, this effort is likely to be associated with diminishing impact, and may indeed not be cost-effective. Furthermore, some of these glycans may have important functions in pigs and therefore knockout may possibly have a deleterious effect. Until then, the effect of antibody binding to the vascular endothelium of the pig graft may be minimized by the transplantation of organs from TKO pigs expressing human complement- and/or coagulation-regulatory proteins, such as the complement regulators CD46, CD55, and CD59, and/or the coagulation regulators thrombomodulin, endothelial protein C receptor, tissue factor pathway inhibitor, and CD39 (2,3,17). The theory is that the presence of the human (transgenic) proteins inhibits the effect of even low levels of antibody binding to the graft vascular endothelium, perhaps preventing activation of the endothelium by complement or coagulation factors, and protecting the endothelium from the effects of complement and/or dysfunctional coagulation/thrombosis.

Furthermore, Lin et al demonstrated *in vitro* that exposure of human platelets and monocytes to porcine aortic endothelial cells (*in the absence of serum antibodies*) can induce procoagulant tissue factor expression on the platelets and monocytes (38). In the GTKO pig-to-baboon kidney transplant model, Lin et al also demonstrated that activation of platelets and monocytes to express tissue factor was associated with the initiation and onset of consumptive coagulopathy, in the absence of an elicited antibody response, and with minimal antibody deposition in the graft (as demonstrated by immunohistochemistry) (15). Such a mechanism could potentially lead to the development of a thrombotic microangiopathy that would appear to be *independent* of serum antibody binding. Whether this activation of tissue factor occurs if the porcine vascular endothelial cells are from a TKO pig is as yet unknown, but in this respect it is notable that the upregulation of tissue factor expression on platelets was similar whether the platelets were exposed to wild-type, GTKO, or GTKO/CD46 pig cells (39). Unless the mechanism of the direct procoagulant effect of platelets and monocytes on vascular endothelial cells (38) can be elucidated and prevented, protection of an organ by expression of one or more coagulation-regulatory proteins may be very important.

In addition to antibody, complement, and platelets, cells of the innate immune system, e.g., neutrophils, natural killer cells, macrophages, may be playing a significant role. At present, their respective roles remain unclear, although natural killer cells may be adding to the inflammatory response to a pig xenograft. In addition, innate immune cells may be promoting/augmenting the adaptive immune response.

## Conclusions

What can be concluded from the data available to date? Current studies suggest that the major prerequisites for pig xenograft survival are (i) a low level of (or, preferably, no) pre-existing natural anti-pig antibody, (ii) prevention of an elicited antibody response (secondary to efficient inhibition of a T cell response), and possibly (iii) prevention of an inflammatory response (40–42). To achieve these goals will require a combination of genetic engineering of the pig and effective immunosuppressive and anti-inflammatory regimens.

An important point that has to be addressed is whether the current methods of measuring anti-pig antibody actually detect *all* antibody. Different methods, e.g., different target cells, can generate variable results, even using the same serum sample (43, and Zhang Z, et al, *Xenotransplantation*, in press). For a clinical trial, if the absence of antibody is considered essential, this may need to be confirmed using various different assays.

However, there is every prospect that, if immunosuppressive therapy that successfully prevents a T cell-mediated, elicited antibody response is administered, some patients with no or low levels of antibody will do well after receiving a TKO pig organ graft. Others may require grafts from TKO pigs in which some SLA antigens have also been deleted (or replaced by HLA). Still others with higher levels of anti-pig antibody (particularly of IgM, making the graft more susceptible to complement-mediated hyperacute rejection) are likely to require the transplantation of TKO grafts that are additionally protected by the expression of one or more human complement- and/or coagulation-regulatory proteins (or by expression of other transgenes with specific functions).

It should also be noted that a further benefit of these genetic engineering approaches appears to be that deletion of an antigenic target, e.g., Gal, or the expression of a human complement-regulatory protein, e.g., CD46, both result in some reduction in the T cell response to the graft (44,45), which in turn can mitigate T cell-dependent B cell activation and antibody production.

It could reasonably be argued that, if there is no evidence that expression of these human (transgenic) proteins is detrimental to the pig or to the recipient, e.g., by acting as receptors for certain viruses and microorganisms, such as measles (46), then their expression in *all* pig grafts will provide another level of protection that may prevent or at least delay the development of chronic rejection (e.g., graft atherosclerosis), which remains a significant problem today in patients with long-term *allografts*. Expression of a human complement-regulatory protein in the pig organ may be particularly important in patients with high levels of anti-pig IgM associated with T cell-independent B cell proliferation/activation (even if they have no anti-pig IgG). Furthermore, not all complement activation is antibody-mediated, and so the expression of a human complement-regulatory protein might be protective against activation of the alternative complement pathway, e.g., associated with ischemia-reperfusion, to which all xenografts will be exposed.

The expression of a human coagulation-regulatory protein might prevent any procoagulant effect from the expression of tissue factor on the donor vascular endothelial cells or on the recipient's platelets and innate immune cells (after exposure to pig vascular endothelial

cells), as reported by Lin (15,38), and this may prolong graft survival. However, if the mechanism by which human platelets, and possibly innate immune cells, activate pig vascular endothelial cells in the absence of serum antibody can be determined, the expression of a human coagulation-regulatory protein may prove less necessary.

Eventually, using genetic engineering techniques, it seems likely that it will be possible to provide a graft (that does not express any antigens against which the patient has pre-existing antibodies) for any patient in need of an organ transplant (even patients who are highly-sensitized to HLA). Long-term graft survival in successfully-immunosuppressed patients would therefore be anticipated, and perhaps further increased by the expression of selected human transgenes. In turn, these genetic manipulations, particularly those that lead to ‘compatibility’ of the pig and recipient MHC, will facilitate achieving the ultimate goal, namely the induction of tolerance to pig organs.

The recipient of the graft will need to receive an effective immunosuppressive regimen. It appears that, by increasing the genetic manipulation of the pig organ, conventional immunosuppressive therapy may be sufficient to prevent a T cell response (and thus a T cell-dependent elicited antibody response) (Iwase H, et al, unpublished data). A low level of immunosuppressive therapy would appear essential unless T cell tolerance can be induced by such methods as (i) hematopoietic cell chimerism, or (ii) donor-specific thymus transplantation (47).

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## Abbreviations

<b>CMAHKO</b>	cytidine monophosphate-N-acetylneuraminic acid hydroxylase gene-knockout
<b>Gal</b>	galactose- $\alpha$ 1,3-galactose
<b>GTKO</b>	$\alpha$ 1,3-galactosyltransferase gene-knockout
<b>Neu5Gc</b>	N-glycolylneuraminic acid
<b>NHP</b>	nonhuman primate
<b>TKO</b>	triple-knockout (pigs that do not express the 3 known pig antigens, Gal, NeuGc, or Sd <sup>a</sup> )

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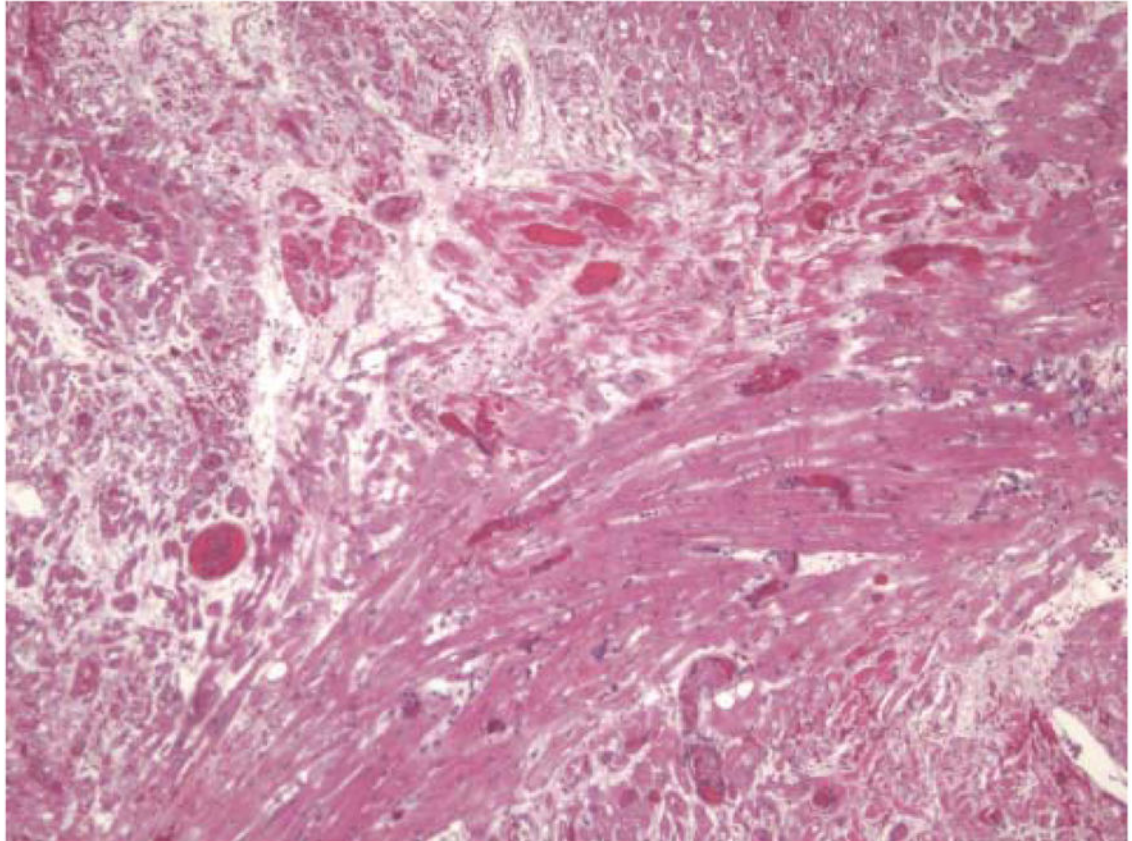


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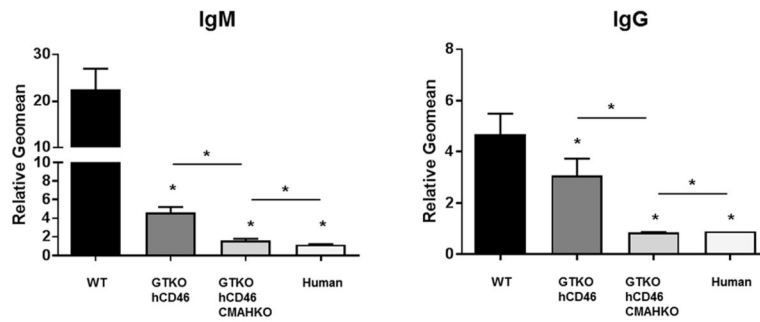
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**Figure 1. Severe thrombotic microangiopathy in a GTKO pig heart that functioned for almost 6 months after transplantation into an immunosuppressed baboon**  
(Reproduced with permission from Tseng Y-L, et al. *Transplantation* 2005;80:1493–1500)



**Figure 2. Human serum IgM (left) and IgG (right) binding to aortic endothelial cells (AECs)** AECs were cultured from a wild-type (WT) pig, an  $\alpha$ 1,3-galactosyltransferase gene-knockout (GTKO) pig expressing the human complement-regulatory protein, CD46, and a GTKO/CD46 pig that did not express NeuGc (GTKO/CD46/CMAHKO). Human IgM and IgG binding to GTKO/CD46/CMAHKO pig cells is almost at the level of binding to human aortic endothelial cells. (\* =  $p < 0.05$ ) (Modified from Lee W, et al. *Xenotransplantation* 2016;23:137–150)

**Table 1**  
Graft or recipient survival after genetically-engineered pig heart or kidney transplantation in NHPS

Year (Ref)	Recipient (n)	Anti-pig Ab	Pig Organ	Survival (days, unless stated)
2005 (6KK)	Baboon (8)	Low	GTKO heart	>16,>23,>56,59,67,78,110,179d (median 63)
2005 (8KY)	Baboon (11)	Low	GTKO kidney	4,13,16,18,26,31,33,56,68,81,83d (median 31)
2009 (13ME)	Baboon (9)	Variable	GTKO heart	<1,1,6,6,7,12,12,35,56d (median 7)
2009 (13ME)	Baboon (3)	Variable	GTKO kidney	2,5,12d (median 5)
2010 (14CL)	Baboon (6)	Variable	GTKO/hCD46 kidney	2,4,9,10,10,16d (median 9.5)
2015b (15HI)	Baboon (7)	Variable	GTKO/hCD46/CD55 heart	15,18,23,33d (median 20.5)
2015b (15HI)	Baboon (7)	Variable	GTKO/hCD46/TBM heart	52,99,130d (median 99)
2015a (2HI)	Baboon (1)	High IgM/low IgG	Multiple GE kidney	136d
2015 (1LH)	Rhesus (2)	Low	GTKO/hCD55 kidney	>126, >133 (>6,>10m) <sup>f</sup>
2015 (1LH)	Rhesus (1)	High	GTKO/hCD55 kidney	6d
2017 3(HI)	Baboon (2)	Low	GTKO/CD46/TBM kidney	12,12d
2017 (3HI)	Baboon (2)	Low	Multiple GE kidney	>7m, >8m

<sup>f</sup> Final outcome unpublished (Adams A, personal communication)

GE = genetically engineered; TBM - thrombomodulin