

CHAPTER 6

TRANSGENIC ORGANS AND XENOTRANSPLANTS

Cristina Costa Vallés¹ and Rafael Máñez Mendiluce^{*,2}

¹*Bellvitge Institute for Biomedical Research (IDIBELL), L'Hospitalet de Llobregat, Spain;* ²*Bellvitge University Hospital, L'Hospitalet de Llobregat, Spain*

**Corresponding Author: Rafael Máñez Mendiluce—Email: rmanez@csub.scs.es*

Abstract: Advances in immunosuppressive treatments reached in the last decades of the 20th century have made solid organ transplantation the treatment of choice for cases of irreversible organ failure. However, the availability of human cadaver organs is limited and the demand for transplants is still on the rise. Also, there is a recognised lack of cells and human tissues for generalised use in transplantation for the treatment of diseases that are characterised by failure of specialised cells (such as pancreatic cells to cure diabetes). Xenotransplantation, which is the transplant of cells, tissues or organs from other species, became the focus of attention in the nineteen-nineties as a solution to the lack of organs and tissues for transplantation. Previous clinical studies using nonhuman primates produced poor outcomes (survival from days to a few months) and confirmed the difficulty of obtaining organs from these species. Since then, progress in xenotransplantation has been slow and still now various immunological and non-immunological barriers need to be overcome. These barriers are reviewed in this chapter and the various approaches explored to date to overcome them, in particular those based on the genetic modification of pigs. Also, cell transplant studies such as those of pancreatic islets in monkeys have led to even more hopeful results. The range of possibilities offered by this technology will be unlimited, making it possible for xenotransplantation to be a clinical reality in a not very distant future.

INTRODUCTION

Advances in immunosuppressive treatments reached in the last decades of the 20th century have made solid organ transplantation the treatment of choice for cases of irreversible organ failure. However, the availability of human cadaver organs is limited

and the demand for transplants is still on the rise. In Spain, the world leader in cadaver organ donation, the greatest achievement has been to avoid a year-on-year growth in waiting lists, as occurs in most countries. It has not been possible to reduce them, despite the notable increase of live donor transplantation; rather these have only served to slow the growth of waiting lists, not to mention the risks of live donor transplantation for the donor. Also, there is a recognised lack of cells and human tissues for generalised use in transplantation for the treatment of diseases that are characterised by failure of specialised cells (such as pancreatic cells to cure diabetes).

Xenotransplantation, which is the transplant of cells, tissues or organs from other species, became the focus of attention in the nineteen-nineties as a solution to the lack of organs and tissues for transplantation. At that point, after the production of the first transgenic pigs, this species was selected as the best source of organs and xenogeneic tissues.¹⁻³ Previous clinical studies using nonhuman primates produced poor outcomes (xenograft survival from days to a few months) and confirmed the difficulty of obtaining organs from these species. Moreover, the risk to public health of using the organs from nonhuman primates was quite high, as vividly demonstrated by the AIDS epidemic. On the other hand, pigs are domesticated animals, which produce large litters, and that, apart from their interest in the food industry, have also had some medical uses (production of insulin, heart valves). Finally, they combine a physiology and anatomy that is similar to primates and they can be genetically modified. Thus, it is not surprising that the possibility of “humanising” pig organs and tissues should be talked about with a view to using them in clinical practice.

Since then, significant advances have been made. The most well studied pig organs for their potential for xenotransplantation are the kidney and heart, followed by the lung and liver. Indeed, the liver is considered as the potential bridge for allotransplantation, either through transplant or by *ex vivo* perfusion. Research is also being carried out on cell xenografts of pancreatic islets, hepatocytes, chondrocytes and neural cells, among others. Despite the great impact that their clinical application would represent, progress in xenotransplantation has been slow, mainly due to technological problems. A decade later, we are still facing various barriers that prevent the clinical application of pig organs, tissues and cells. These barriers are reviewed below and the various approaches explored to date to overcome them, in particular those based on the genetic modification of pigs, are described.

OBSTACLES FOR CLINICAL XENOTRANSPLANTATION

Immunological Barriers

The main obstacle for xenotransplantation to be used in clinical practice is the strong immune response caused by the pig organ in the recipient.³ Cell, tissue and organ xenografts are subject to a variety of rejection mechanisms that include humoral and cellular immune responses. The cellular immune response seems to play a key role in the rejection of cell grafts, such as hepatocytes and pancreatic islets, while rejection of vascularised organs mainly involves humoral immunological mediators. Various types of rejection have been described in solid organ xenotransplantation, depending on the time elapsed following the transplant and the immune elements involved: Hyperacute, acute humoral and acute cellular rejection.¹⁻³

Hyperacute rejection (HAR) is the first-type to occur between minutes and hours after the xenotransplant. HAR is triggered by a humoral immune response in which xenoreactive natural antibodies (XNA) (already present in the recipient) are deposited on the endothelium of the xenograft, activating the complement system and causing fluid extravasation from the intravascular space into the interstitium (oedema).¹⁻³ This process also causes the activation of the coagulation cascade and thus thrombosis, ischemia and necrosis of the xenograft in a short period of time following the transplant. The main xenoepitope recognised by XNA is the disaccharide galactose- α -1,3-galactose (Gal), widely expressed in pig tissues and synthesised by the α 1,3-galactosyltransferase (α 1,3-GT) enzyme. Humans and Old World primates lack functional α 1,3-GT and have high titre of anti-Gal antibodies.

Acute humoral xenograft rejection (AHXR), also known as acute vascular rejection or delayed xenograft rejection occurs in a period of days to months after the transplant. It has not been possible to prevent it using currently available immunosuppression regimens.¹⁻⁴ AHXR shows notable similarity to HAR since it involves a strong humoral immune response with the participation of antibodies, the deposition of complement proteins and thrombosis in xenografts. However, in other respects AHXR differs significantly from HAR, especially in the origin of the antibodies responsible for rejection. In this case, it involves a response in which anti-Gal antibodies participate, but it also includes antibodies targeting other epitopes. Further, AHXR is characterised by the presence of innate immunity cell infiltrates (NK cells and macrophages) and an activation of endothelial cells that promotes intravascular thrombosis and fibrin deposition.

Acute cellular rejection occurs some days after the transplant and is a response mediated mainly by T cells against the donor antigens. The activation of these cells during xenotransplant rejection is mediated by a primary signal through the T receptor and secondary costimulatory signals conserved across species.¹ Currently, it is thought that it is possible to control the cellular immune response against the xenotransplant using the various immunosuppressive protocols currently available, since no failure of pig xenograft caused by this type of rejection has been demonstrated. However, this point cannot be properly judged until AHXR can be prevented in an effective and systematic way.

Avascular tissue and xenogeneic cells are also subject to rejection of the graft, similar to the process that occurs in solid organ rejection, but without the vascular component and with special features for each cell and tissue.⁵ Pancreatic islets have drawn great attention due to their potential clinical application for the treatment of diabetes. First, adult pig beta cells do not express the Gal antigen, thus reducing the humoral component of rejection. Potent immunosuppressive protocols focussed on averting the activation of T cells have led to long-term survival of pig pancreatic islets in diabetic monkeys (>140 days).⁶ The problem for the clinical application of cellular xenotransplantation lies in the fact that, while they present fewer immunological barriers than solid organs, they still require strong immunosuppression of the recipient that cannot be sustained indefinitely. In any case, the strategies developed for this type of transplant may be very useful for their application in other organs of tissues of interest, especially regarding genetic modification of the donor pig that can involve a wide variety of cell and tissue types. Therefore any progress made in this area has an impact that goes beyond xenografts, be it to cells, tissues or organs.

Strategies to Prevent Hyperacute Rejection

The various techniques have been developed to prevent HAR can be summarised in two groups: Those focused on modifying the xenograft, and those that use systemic treatments to alter the immune response of the recipient. The most refined methods are based on the genetic modification of pigs used as the source for organs, since they are less harmful for patients and their objective is to decrease the need for immunosuppression or other conditioning treatments. However, these are complex techniques and involve slow and expensive procedures. Systemic treatments, on the other hand, may help us to identify molecules or key rejection processes and to speed up the clinical application of xenotransplants. Specifically, the key roles of XNA and complement in HAR were confirmed by the effectiveness of antibody absorption by plasmapheresis and systemic complement inhibition to prevent this type of rejection.

The first approach to neutralise HAR by genetic engineering was the inhibition of complement activation through the expression of human complement regulatory proteins in transgenic pigs.^{7,8} The reason for this was the notable restriction of the function of the complement regulatory proteins between species, which led to the suggestion that pig molecules were unable to control the activation of the human complement. Subsequent studies showed that pig complement regulatory proteins were also able to regulate, at least in part, the activity of the human complement.⁹ This suggests that the benefit of the expression of the human complement proteins in pig cells is not only due to specificity, but also to the increase in expression of these proteins.

Initially in vitro studies demonstrated that the expression of human CD59 (hCD59) or CD55 (hDAF) in pig cells notably protects these cells from cytolysis triggered by the human serum. Ex-vivo perfusion experiments with human blood also demonstrated that kidneys and hearts from pigs transgenic for human CD59 functioned for longer than control animals. Later it also demonstrated the almost systematic prevention of HAR and longer survival rates of transgenic pig organs that expressed human complement inhibitors in transgenic pig-to-primate models.^{2,10} However the use of organs from these transgenic pigs did not achieve full protection against humoral xenograft rejection, since all the organs were subject to AHXR sooner or later, the various immunosuppressive protocols being unable to modify this response (Tables 1, 2 and 3). This led research to focus on the production of pigs with genetic modifications that would reduce the reactivity of the organs to xenoantibodies present in human serum.

Before homologous recombination and “knockout” techniques were available in pigs, one of the most widely investigated strategies was based on the transgenic expression of human alpha 1,2-fucosyltransferase (H transferase, HT).¹¹ HT produced fucosyl residues (H antigen of the O blood group) that are universally tolerated (Fig. 1). HT was shown to compete efficiently with alpha1,3-GT for the same substrate, N-acetyl-lactosamine, preventing the transfer of the terminal galactose, the residue which gives rise to the production of the Gal antigen. The reduction in the expression of the Gal epitope in HT transgenic pig cells leads to a decrease in their reactivity with human antibodies and in cytolysis caused by human sera.¹¹ Further, the hearts of HT-transgenic mice have been shown to have higher survival rates after being perfused with human serum or transplanted to 1,3GT knockout mice, which produce anti-Gal antibodies. As HT and other competitive enzymes are not able to completely inhibit the expression of Gal epitopes, this technique was combined with the expression of human complement inhibitors. Thus, peripheral blood mononuclear cells and aortic

Table 1. Orthotopic kidney transplant of genetically modified pigs in cynomolgus monkey

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Zaidi/1998	hDAF-tg + CsA + CyP + CS	7	13 days (6-35)
Cozzi/2000	hDAF-tg + CsA + CyP + CS + Spx + EPO	9	35.2 days (5-78)
Vangerow/2001	hDAF-tg + CsA + CyP + CS	4	11.5 days (9-15)
	hDAF-tg + CsA + CyP + CS + C1-INH	4	33.7 days (18-68)
Cozzi/2003	hDAF-tg + CsA + CyP + CS + Spx + EPO + MPS	10	24.1 days (2-51)
Cozzi/2003	hDAF-tg + CsA + CS + Spx + MPS + MTX	4	26.7 days (16-41)
Lam/2005	hDAF-tg + CsA + CyP + CS + MPS + GAS914	4	21.5 days (6-37)
	hDAF-tg + CsA + CyP + CS + MPS + GAS914 + sCR1	2	12.5 days (10, 15)
	hDAF-tg + CsA + CyP + CS + MPS + GAS914 before Tx + sCR1	4	24.7 days (10-37)

hDAF-tg, kidney of transgenic pig expressing hDAF; CsA, Cyclosporin A; CyP, cyclophosphamide; CS, corticosteroids; Spx, splenectomy; EPO, erythropoietin; C1-INH, C1 inhibitor; MPS, sodium mycophenolate; MTX, methotrexate; sCR1, soluble complement receptor-1; Tx, transplant.

endothelial cells of double transgenic pigs that co-express HT and hCD59 are better protected from lysis by human serum than controls or single transgenic cells for each of the genes.¹² In addition, double transgenic cells maintained their resistance to XNA and complement after being treated for 24 hours with porcine proinflammatory cytokines.¹² Despite these advances, these pigs were developed to be used in cellular xenotransplantation and the organs have not been transplanted to nonhuman primates, so it has not been possible to study their effectiveness in this experimental model in comparison to pig organs carrying other types of genetic modifications.

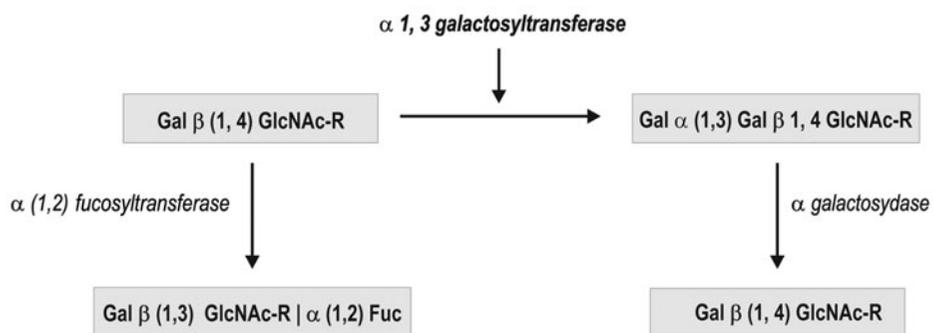


Figure 1. Biosynthesis of galactose $\alpha 1,3$ Galactose (Gal $\alpha 1,3$ Gal) and their inhibitors. The enzyme $\alpha 1,3$ -galactosyltransferase adds galactose to the N-acetyllactosamine (Gal $\beta 1,4$ GlcNAc) to form Gal $\alpha 1,3$ Gal. Transgenesis of the enzyme $\alpha 1,2$ - fucosyltransferase produces a fucosylated structure (antigen H; blood group O), reducing the expression of Gal $\alpha 1,3$ Gal. Treatment with the enzyme α -galactosidase removes the terminal α -galactosyl residues of adjacent carbohydrates, preventing the expression of Gal $\alpha 1,3$ Gal.

Table 2. Orthotopic kidney transplants from genetically modified pigs to baboons

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Lawson/1997	hDAF and hCD59-tg + CsA + CyP + CS	6	7.5 days (<10)
Diamond/1997	hDAF and hCD59-tg + CsA + CyP + CS + MPS	7	7.6 days (<15)
Cowan/2000	hDAF, hCD59 and HT low-tg + LMWH	6	4 days (3-5)
Buhler/2001	hDAF-tg + IA + CVF + ATG + CyP + CS + MMF + PGE + aCD154 + Spx + TmI + Heparin	3	28.7 days (28-29)
Cowan/2002	hDAF, hCD59 and HT low-tg + ATIII ± LMWH	4	5 days (4-6)
Ghanekar/2002	hDAF-tg + CsA + CyP + CS + GAS914 + RAD	2	28 days (20, 36)
	hDAF-tg + CsA + CS + GAS914 + RAD + RATS	4	23 days (20-26)
	hDAF-tg + CsA + CS + GAS914 + RATS	3	20 days (18-22)
Zhong/2003	hDAF-tg + CsA + CyP + CS + RAD low	4	18.5 days (4-40)
	hDAF-tg + CsA + CyP + CS + RAD low + GAS914 low	4	19.2 days (10-37)
	hDAF-tg + CsA + CyP + CS + RAD high + GAS914 high	4	14.5 days (9-25)
	hDAF-tg + CsA + CyP + CyP + CS + RAD high + GAS914 high	4	18.7 days (7-36)
Barth/2003	hDAF-tg + IA + CVF + ATG o Tmx + CyP + CS + MMF + PGE + aCD2 + aCD154 + Spx + TmKid	5	24.4 days (18-30)
Ashton-Chess/2003	hDAF-tg + IA + CsA + CyP + CS + MPS	4	6 days (5-9)
	hDAF-tg + CsA + CyP high + CS + MPS	4	9.2 days (7-12)
	hDAF-tg + IA + CsA + CyP high + CS + MPS	4	7 days (5-10)
Ashton-Chess/2004	hDAF-tg + CsA + CyP + CS + MPS	2	9 days (9)
	hDAF-tg + CsA + CyP alto + CS + MPS + Mx	4	8 days (6-10)
González Martín/2004	hDAF-tg + CsA + CyP + CS + MPS + GAS914	10	7 days (1-31)
	hDAF-tg + CsA + CyP + CS + GAS914 + FTY720	3	8 days (4-28)
	hDAF-tg + CsA + CS + GAS914 + FTY720 + Bas	3	8 days (3-13)
	hDAF-tg + CS + GAS914 + FTY720 + Bas + RAD	4	9 days (1-20)

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Table 2. Continued

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Ménoret/2004	hDAF and hCD59-tg	2	5.5 days (5, 6)
Yamada/2005	Gal KO + CVF + ATG + anti-CD2 + anti-CD154 + MPS + CS + Spx + Tmx ± WBI	3	29 days (20-34)
	Gal KO ± CVF + ATG + anti-CD154 + MPS + CS + Spx + Tmx ± WBI + VTL	6	34.1 days (4-68)
	Gal KO ± CVF + ATG + anti-CD154 + MPS + CS + Spx + Tmx ± WBI + VTL + TmKid	5	44.8 days (16-83)
Chen/2005	Gal KO + ATG + FK506 low + MMF + CS low	3	9.7 days (8-10)
	Gal KO + CVF + ATG + FK506 + MMF + CS	3	12.7 days (9-16)
Chen/2006	hDAF-tg + RATS o ATG + FK506 + CS + GAS914 o TPC + MMF	5	23.8 days (7-75)
	hDAF-tg + CVF + RATS o ATG + FK506 + CS + GAS914 o TPC + LF + DSG + aCD20	2	11 days (8, 14)

hDAF-tg, kidney of transgenic pig expressing hDAF only or other transgenes as indicated; Gal KO, knockout for α 1,3-GT; CsA, cyclosporin A; CyP, cyclophosphamide; CS, corticosteroids; LMWH, low molecular weight heparin; ATIII, antithrombin III; MMF, mofetil mycophenolate; MPS, sodium mycophenolate; IA, immunoglobulin immunoabsorption; CVF, Cobra venom factor; PGE, prostacyclin PGE2; RAD, rapamycin derivative; ATG, antithymocyte globulin; RATS, rat anti-thymocyte serum; aCD154, anti-CD154 mAb; Mx, mitoxantrone; Bas, basiliximab or anti-IL2R mAb; Spx, splenectomy; Tmx, thymectomy; WBI, whole body irradiation; VTL, vascularised thymic lobes; TmKid, thymus-kidney; LF, LF15-0195; DSG, analogue of 15-deoxyspergualin; aCD20, anti-CD20 mAb.

The description of the process of nuclear transfer of a somatic cell into a germinal cell by the team led by Ian Wilmut in 1996 opened the door to the generation of “knockout” pigs. From that moment, several groups started the race to produce the first α 1,3-GT deficient pigs. There was a concern that pigs produced by this technique would not be viable, but the first litters of α 1,3-GT “knockout” pigs were healthy and developed well.¹³ Subsequent studies showed survival times of up to 11 days for kidneys of α 1,3-GT knockout pigs transplanted in baboons, meaning that the organs of these transgenic pigs were also protected against HAR,¹⁴ as occurred in animals transgenic for human complement regulatory proteins. Despite this progress, the majority of human sera show reactivity towards pig cells that lack for α 1,3-GT gene, suggesting the existence of antibodies that recognise other antigens apart from the Gal epitope.¹⁵ Therefore, the introduction of a human complement inhibitor is still necessary to completely block human serum-mediated cytotoxicity.

Another carbohydrate in pigs of interest to prevent HAR in organ xenotransplantation is the antigen of the Type A blood group. As in the human, these antigens are present in some pigs and can be recognised by human antibodies directed against this blood-type. The reactivity of human serum IgM against this epitope was discovered in a kidney

Table 3. Orthotopic heart transplants from genetically modified pigs to baboons

First Author/Year	Treatment	Number of Cases	Mean Survival (Range)
Schmoeckel/1998 and Goddard/2002	hDAF-tg + CsA + CyP + CS + MMF	10	11.7 days (1-39)
Brenner/2005	hDAF-tg + CsA + CyP + CS + MPS	3	14.6 days (11-20)
Brandl/2005 and Brandl/2007	hDAF-tg + ATG + FK506 + CS + GAS914 low	2	5 days (1, 9)
	hDAF-tg + ATG + FK506 + CS + GAS914 high + aCD20	1	30 hours
	hDAF-tg + ATG + FK506 + CyP + CS + GAS914 high	3	13.3 days (1-25)
	hDAF-tg + ATG + FK506 + CyP + CS + GAS914 high + aHLA-DR	5	1.8 days (0, 2-4)

For these calculations the authors excluded cases of loss of organ due to technical failure and one case of HAR in the study of Goddard et al. hDAF-tg, kidney of transgenic pig expressing hDAF; CsA, cyclosporin A; CyP, cyclophosphamide; CS, corticosteroids; MMF, mofetil mycophenolate; MPS, sodium mycophenolate; aCD20, anti-CD20 mAb; aHLA-DR, anti-HLA-DR mAb.

from a Type A group pig whose kidneys were extracorporeally connected to a volunteer dialysis patient. Nevertheless, these antibodies should not be an obstacle to clinical xenotransplantation, since blood groups can be selected for the animals used as source of organs, in the same way as with allotransplantation.

Strategies to Prevent or Treat Acute Humoral and Cellular Xenograft Rejection

The methods described above, alone or in combination, have successfully and routinely manage to prevent HAR. However the same strategies have not proved successful to avoid AHXR. The studies carried out to date suggest that this type of rejection is not caused by a single immunological element, but a collection of them. We will go on to describe research carried out so far into the control of humoral as well as cellular xenograft rejection. It is difficult to separate these two processes as they overlap in the time of progression and possibly also in the immunological response mechanisms. However, it should be highlighted that cellular rejection has not been an insurmountable obstacle and that the various immunosuppressive protocols investigated have managed to prevent this type of rejection systematically.

Most of the information currently available on preclinical xenotransplantation comes from studies using hDAF transgenic pigs as a source of organs and cynomolgus monkeys or baboons as recipients. Results of these studies are summarised in the Tables 1 to 3, focusing on orthotopic transplants of kidney and heart from genetically modified pigs, given their particular preclinical importance. The transgenic expression of hDAF, alone or in combination with hCD59, protects against HAR and confers some protection against HAXR.^{16,17} One aspect that is worth highlighting is that the species of recipient primate also seems to influence the survival of the xenograft, survival rates being higher in cynomolgus monkeys than in baboons, perhaps due to the fact that the latter being a model that is closer to humans (Tables 1 and 2). Without immunosuppression AHXR

occurs 4 or 5 days after the transplant of hDAF transgenic organs in baboons,¹⁸ there being no similar results from cynomolgus monkeys. The use of an immunosuppressive regimen that includes cyclophosphamide (CYP), cyclosporin (CsA) and corticosteroids (CS) increases the mean survival of the renal transplant recipient to 7.5 days in baboons and approximately 12 days in cynomolgus monkeys.^{2,10} Parallel studies of orthotopic heart xenotransplants in baboons show mean survival rates of 12-15 days in similar conditions of immunosuppression. The addition of other immunosuppressants, such as rapamycin, mycophenolate mofetil or methotrexate, to the aforementioned protocol and performing splenectomy during the transplantation procedure, increased the mean survival rates for renal xenografts to up to 25-35 days in cynomolgus monkeys and to 9-20 days in baboons^{16,17} (Tables 1 and 2). The temporary use of systemic complement inhibitors such as C1 inhibitor or soluble complement receptor 1 (sCR1, TP10), was also found to be effective, both in prolonging kidney xenograft survival and to revert AHXR, once diagnosed.¹⁹ However, the toxicity of these products in the form of increased susceptibility to infections does not allow a long-term treatment with complement inhibitors.

Initially it was thought that anti-Gal antibodies also have an important role in AHXR, which led to the development of a series of polymers containing many Gal epitopes, following the failure of repeated immunoadsorption for neutralising these antibodies.¹⁸ The most widely tested was GAS914, a trisaccharide composed of α 1,3 Gal with a molecular weight of 500 kDa.²⁰ Using injections of GAS914 it is possible to continuously neutralise anti-Gal antibodies (Fig. 2). This also reduces the intensity

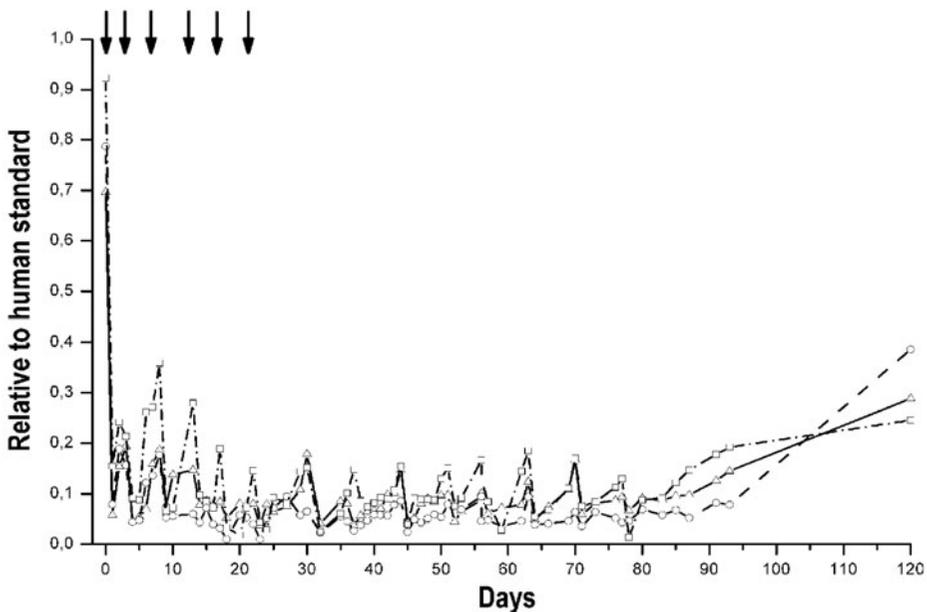


Figure 2. Continuous depletion of natural anti-Gal with GAS914 in baboons. The arrows indicate the days on which GAS914 was injected, with black corresponding to doses of 5 mg/kg and grey to 1 mg/kg. The levels of anti-Gal (circles), anti-Gal IgG (triangles) and porcine haemolytic (squares) antibodies are shown compared to a standard human serum, from a pool of 50 different human sera, which has been arbitrarily assigned a value of 1.

of the AHXR, although without improving xenograft survival even with the addition of potent immunosuppressive treatments.²¹ The presence of antibodies in AHXR despite continuous depletion of anti-Gal antibodies in the blood suggested that the rejection was caused by antibodies directed against other pig epitopes. These results have later been confirmed with the production of $\alpha 1,3$ -GT “knockout” pigs, that do not express the antigen Gal. Kidneys transplanted from these gal-deficient pigs into baboons have reached mean survival rates of 10 days under mild immunosuppression, and using higher-dose immunosuppression a mean of 29 days and a maximum of 34 days.^{15,22} However these organs also suffer from AHXR mediated by antibodies other than anti-Gal that cannot be avoided with immunosuppressive treatments.

The best renal xenotransplantation results in baboons have been obtained using kidneys of $\alpha 1,3$ -GT knockout pigs, in combination with protocols that use chimerism to promote graft tolerance.²² Mean survival rates of 44.8 days, with a maximum of 83 days, have been attained by transplanting the pig kidney together with vascularised thymic lobe of the same animal, previously grafted under the renal capsule, with a conditioning regimen that included the temporary depletion of complement and T cells and maintenance with anti-CD154 monoclonal antibodies, mycophenolate mofetil and corticosteroids. The results represent an improvement compared to the mean and maximum survival rates of 24.4 and 30 days, respectively, achieved with similar experiments using kidneys from hDAF- transgenic pigs. This suggested that $\alpha 1,3$ -GT knockout pigs offer advantages with respect to those that are transgenic for complement regulatory proteins in the AHXR, possibly due to the fact that the immune response is not amplified as shown in experimental models in rodents.⁵ However it still remains unknown to what extent the combination of Gal deficiency by “knockout” with the transgenic expression of complement inhibitors would improve xenograft survival, compared to organs that have only one genetic modification.

A feature that has arisen in all the preclinical studies carried out to date is that treatments that inhibit the production of antibodies, such as high doses of cyclophosphamide or anti-CD154 monoclonal antibodies, do decrease or prevent AHXR. However, they all are very aggressive and lead to excessive immunosuppression, accompanied by severe side effects (gastrointestinal lesions, anaemia, infections, etc.) which in themselves jeopardise the life of the recipient. For this reason, the experiments carried out by the group led by Dr David Cooper are of great importance. These researchers performed a heterotopic heart xenograft transplantation in baboons from $\alpha 1,3$ -GT knockout pigs, using immunosuppressive levels that were acceptable for clinical practice.²³ Most of these hearts were rejected with signs of AHXR or thrombotic microangiopathy. Nonetheless, they reached average and maximum survival times of 78 days and 6 months respectively, the longest survivals of porcine xenografts in nonhuman primates described to date.²³

The thrombotic microangiopathy described in these experiments is considered to be another manifestation of AHXR, closely linked to the antibody-mediated response. Thus, recipients dying after long survival times due to causes not associated with rejection are found to have xenografts with minimal or non-existent pathological findings. For this reason, although it is not possible to completely rule out an effect of clotting incompatibilities between pigs and humans, as we will see in the following section that looks at the physiological barriers between these species, the control of the response mediated by anti- nonGal antibodies has become the biggest challenge for clinical xenotransplantation. Our group has preliminary data suggesting that these antibodies

are not directed against porcine proteins, but rather the targets, though not the Gal antigen, are also carbohydrates. Although it would be reasonable to expect that the antibodies are directed against many epitopes, their characterization would open the possibility of developing new treatments to prevent or treat AHXR. Given that one of the principal advantages of xenotransplantation is the possibility of modifying the donor organ, the solution for AHXR would come from genetically engineering the donor animal, to avoid the expression of those elements that cause a uncontrollable production of xenoantibodies.

Together with these measures intended to decrease the reactivity of anti-nonGal xenoantibodies, and potential clotting incompatibilities, other genetic modifications might decrease the immunogenicity of pig organs. Key potential targets are the immunological responses induced by the porcine cells, such as those mediated by the CD80-CD86/CD28 pathway, which is conserved across the swine-to-human species barrier. Specifically, CD86 expressed in porcine aortic endothelial cells provides strong costimulatory signals to human T and NK cells.^{1,24} Porcine CD86, in contrast to its human counterpart, is expressed in a wide variety of cells and tissues, and the signal mediated by CD28 is resistant to immunosuppression by calcineurin inhibitors. Other elements which have a potential role in therapeutics are cytokines such as TNF α and β . The use of strategies that block the TNF may be useful in the development of xenografts resistant to AHXR, as has been demonstrated in rodent xenotransplantation models.²⁵ In general, the objective would be for these techniques to allow clinical xenotransplantation using the minimum possible level of immunosuppression in the recipient.

Non-Immunological Barriers

Physiology of the Xenograft

It is well established that xenogeneic proteins such as porcine insulin can work correctly in humans. However, it is not clear whether xenografts are able to perform their functions in an environment other than that for which they have been genetically programmed, and, if so they are, for how long this functioning can be sustained. Pig kidneys have maintained the life of nonhuman primates for several months,^{16,22} xenograft function apparently failing due to rejection rather than to the existence of physiological incompatibilities between the species. Recipients of α 1,3-GT knockout kidneys required continuous treatment with human albumin to maintain protein levels within the normal range,²² as a consequence of the proteinuria produced after the transplant and that continued throughout the three months that the xenograft survived. The clinical symptoms of the proteinuria (oedema) were different depending on the immunosuppression protocol used, suggesting that proteinuria was the consequence of the xenograft rejection, and not of a mutual physiological incompatibility. Furthermore, nonhuman primates transplanted with α 1,3-GT knockout kidneys did not suffer from the anaemia previously described with the immunosuppressive protocols that included cyclophosphamide, confirming that it had been a consequence of the treatment toxicity rather than due to the inability of porcine erythropoietin to maintain erythropoiesis.

It has been confirmed that porcine hearts and kidneys are able of maintaining a similar physiology to human for long periods of time and they are candidates for the first solid organ clinical xenotransplants. In contrast, xenografts of other organs such as the lung and liver have not survived more than a few days, though even this has demonstrated that they can maintain the life of the recipient for short periods of time. Nevertheless,

some significant physiological differences can be expected between the donor and the recipient, especially in the case of the liver due to its complex metabolic system.

In the case of porcine heart and kidney, the existence of some long-term minor incompatibilities cannot be ruled out. An example of this is the clotting abnormalities described in AHXR. Currently it seems that these changes may be dependent of the deposit of xenoantibodies in the xenograft, but the existence of some kind of physiological incompatibility cannot be completely ruled out. In vitro, porcine cells have an inherent tendency to clot spontaneously in human plasma, an effect that seems to be dependent on some molecular incompatibilities between porcine and human blood-clotting regulators.²⁶ Specifically, porcine thrombomodulin (a key anticoagulant expressed by endothelial cells) hardly works in the human system.²⁶ If clotting abnormalities after xenotransplantation occurred independently of antibodies, the difficulties found in preventing and treating AHXR in porcine xenotransplants to nonhuman primates would be explained. Nevertheless, accumulated experience suggests that physiology should not be an insurmountable obstacle to clinical xenotransplantation of porcine organs. Also some methods are currently under investigation to solve the potential clotting incompatibilities between pigs and humans, including the genetic engineering of pig organs. If these problems were to persist once the problem of AHXR has been overcome, it is very likely that they could be also treated by the introduction of suitable human genes/proteins to the donor pig.

Risk of Transmission of Infection from the Xenograft

The success of organ xenotransplantation depends on the balance between the immunosuppressive treatment necessary to avoid rejection and the risk of opportunistic infections or cancer such treatment may cause. In the case of xenotransplantation, experimental data available to date suggest that more immunosuppression is required to prevent AHXR, and, therefore, that the theoretical risk of opportunistic infections is greater in xenotransplantation than in allotransplantation. Furthermore, the use of nonhuman cells, tissue or organs will increase the spectrum of opportunistic infections, since it will include diseases from the animal species used as a source of organs. The possibility that a new pathogen agent could be transferred to the recipient through xenotransplantation, and the possible passing of this to the general population, as happened with AIDS, is cause of concern among scientists and those responsible for Public Health.

The terms of “xenosis” and “xenozoonosis” have been proposed to describe infections produced by microorganisms of other animal species, that do not cause infection in humans in normal circumstances, but that might be transferred from a xenograft.²⁷ The probability of a specific microorganism of an animal species causing a disease in humans is unknown. In theory it may be fairly high in the cases of microorganisms that are zoonotic under normal conditions (for example, *Toxoplasma gondii*), similar to others that cause infections in allotransplantation (such as cytomegalovirus, CMV), and capable of infecting a wide spectrum of species (such as *Pneumocystis carinii*), as well as those microorganisms that can replicate in vitro in human cells. However, it is likely that xenosis, also known as xenozoonosis, caused by bacteria, fungi and parasites, arising from both common and species-specific pathogens, do not imply a particular risk for the recipient of the xenograft and even less for public health. The reason for this assertion is that this type of infections should be prevented in the animal that is to be the source of organs.

The production of animals in confined and isolated areas, from which only animals that are negative to all known pathogens are prospectively selected, can minimise the number of infections that these animals carry. Accordingly, it is important that strict protocols are established for clinical and microbiological assessment, using both immunocompetent and immunodepressed animals, to enable animals with any traces of infection to be detected. This applies, be they carriers of latent microorganisms, similar to those that cause infection in allotransplants, or of pathogens of other species. Removal of animals that are infected or are carriers of germs leads to the identification of the so-called specific pathogen free (SPF) animals, the use of which minimises the risk of transmission of both classical zoonosis and those arising from xenosis transmitted by the xenograft. In addition to these causes of exclusion for any animal, specific criteria can be included according to the organ to be transplanted. Thus, the presence of *Mycoplasma sp.* would rule out any animal as potential source for a lung xenograft, or the virus Coxsackie for heart xenografts. The ease of obtaining SPF pigs is another of the advantages of using this species as a source of organs, a process that logistically would be very difficult to carry it out with nonhuman primates.

The risk of transmission of the xenosis may be further reduced by the production of pigs totally free of germs (gnotobiotic). Although there are currently no facilities that allow the production of mammals in this state, their construction is totally feasible, with the high cost being the most significant disadvantage. However, gnotobiotic animals are less robust than those which have been subject to normal microbiological colonisation, and at present they do not seem to offer any advantages over SPF animals in terms of minimising the risk of transmission of xenosis. Therefore, the production of gnotobiotic pigs has been put on hold until clinical experimentation demonstrates its necessity.

With the production of SPF animals, the risk of transmission of diseases would be limited to some pathogens capable of producing latent infections in the source animal and caused by vertically transmitted viruses that cannot be prevented in the source animal by early weaning and/or caesarean birth. All species have viruses that persist within the host cells in a latent state. While the best known are the herpes viruses and retroviruses, these also include the hepatitis viruses, adenoviruses, rabies and pseudorabies viruses, reoviruses, and papovaviruses among others. It is still not known whether nonhuman latent viruses represent an infection and disease risk for humans. In vitro, it has been possible to infect human neurons with the porcine pseudorabies virus and to transmit various viruses between species in experimental models. Nevertheless, it is hard to believe that in the case of a species such as the pig, with which humans have been in contact for many thousands of years, there are many infections with capacity of being transferred from person to person that have not yet manifested themselves. On the other hand, the infection caused by Nipah virus in pig slaughterhouse workers in Asia, after contact with infected animals, and the recent epidemic of Severe Acute Respiratory Syndrome (SARS), caused by a coronavirus, transmitted through the consumption of exotic animals in China, are good examples of the ability these zoonoses to spread.

The potential re-activation of latent herpes virus infections after xenotransplantation of a porcine organ has been subject of particular attention in preclinical research on xenotransplantation. Three herpes viruses have been identified in swine: Porcine cytomegalovirus (pCMV), and porcine lymphotropic herpes virus 1- and 2- (PLHV-1, -2) which, in pigs that have been subject to bone marrow transplantation, are associated with a lymphoid proliferative syndrome similar to post-transplant proliferative disease (PTPD) seen in allotransplantation. Herpes viruses are species-specific so if infection occurs after

the xenotransplant, it should be restricted to the xenograft. The replication of pCMV has been described in porcine xenografts in nonhuman primates, causing an infection that damages porcine endothelial cells and tissues.²⁸ The elimination of pCMV from swine litters has been possible through early weaning of newborn animals, and the absence of pCMV in xenografts has been associated with the reduction of clotting disorders and improvement of survival rates of pig xenotransplants in nonhuman primates. Activation of PLHV-1 has not yet been demonstrated in any solid organ xenotransplant. However, unlike pCMV, this virus cannot be eliminated from source animals by early weaning of newborns, so remains a potential pathogen in porcine organ xenotransplantation.

Other infections that cannot be prevented using SPF pigs are those caused by vertically transmitted viruses. These include porcine endogenous retroviruses (PERV) which are viruses that have been permanently integrated into the genome of the host during the evolution of mammals, and are transmitted vertically from mother to offspring. Although they are not pathogens in the host, these retroviruses can be xenotropic, that is, capable of infecting other species. Two PERV have are known to have the capacity to infect human cells *in vitro*, which leads to us consider the possibility of recombination or complementation of xenograft endogenous retroviruses with viruses present in human tissues, and the potential risk of induction of tumours and immunodeficiencies caused by viruses.

Research undertaken to date, on humans in contact with living pig tissue, workers in pig slaughterhouses, human patients who have received transplants in contact with pigs and nonhuman primates who have received pig organs and severe immunosuppressive treatment, has not shown the existence of PERV replication in humans or nonhuman primates.^{29,30} However work is ongoing to characterise PERV, to optimising systems for their detection, and to develop approaches to avoid or minimise the associated risks. On the one hand, certain families of miniature swine, “mini pigs”, that do not transmit PERV to human cells have been identified recently. On the other hand, transgenic techniques such as siRNA expression technique are being applied to inhibit the expression of PERV,³¹ raising the prospect that this infection may be avoided by manipulating the animals to be used as sources for organs.

Given all this, on the basis of the currently available information, the risk of xenosis, also known as xenozoonosis, should not be an obstacle for clinical xenotransplantation of pig organs. In contrast to the situation existing a few years ago, risks have now been identified, investigated and tests have been developed that enable them to be assessed in preclinical and clinical studies. Although the possibility of an infection arising from the xenograft that would affect public health cannot be ruled out, the risk seems insignificant and avoidable by close supervision of the source animals and recipients of xenografts.

CONCLUSION

Xenotransplantation has great potential to solve the problem of the lack of human tissues and organs for transplantation and continues to be a possible alternative to allotransplantation. Progress has been slow in research in this area due to technological problems, such as the difficulty of producing “knockout” pigs and the evaluation of xenozoonosis risks. However, the key tools have now been established and so the field can now develop much faster.

The main barrier to its clinical application is immune rejection, especially the humoral response triggered by vascularised xenografts. The identification of the key systems and molecules that are involved in the process of rejection, and the development of strategies to overcome them is just a matter of time. The use of porcine organs that have been subject to various genetic manipulations has already shown significant improvements in the xenotransplantation of organs to nonhuman primates. Cell transplant studies such as those of pancreatic islets in monkeys have led to even more hopeful results. Then the range of possibilities offered by this technology will be unlimited, making it possible for xenotransplantation to be a clinical reality in a not very distant future.

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