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XENOTRANSPLANTATION - STATE OF THE ART

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1. ABSTRACT

Organ transplantation is limited by the number of cadaveric human donor organs that become available. Xenotransplantation - the transplantation of organs and tissues between animal species - would supply an unlimited number of organs and offer many other advantages. The pig has been identified as the most suitable donor animal. Pig organs, when transplanted into humans or nonhuman primates, are, however, rejected hyperacutely within minutes by antibody-mediated complement activation. Human anti-pig antibodies have been identified as being directed against Galα1-3galactose epitopes on pig vascular endothelium. Major efforts are being made to overcome this hyperacute rejection. Methods being investigated include (i) depletion or inhibition of recipient antibodies or complement, (ii) development of transgenic pigs that do not express the αGal epitope and/or express a human complement inhibiting protein (*e.g.* DAF), and (iii) development of immunological tolerance to pig organs in the recipient. If complement activation is prevented, *e.g.* by inhibition of complement activation by cobra venom factor, soluble complement receptor 1 or by the use of hDAF transgenic pig organs, then "delayed xenograft rejection" occurs and is again believed to be largely antibody-dependent. Experimental pig-to-primate organ xenotransplantation is now, however, resulting in transplant function for days and weeks rather than minutes, and there is therefore optimism that we are on the threshold of a new era in the field of the transplantation of vital organs.

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2. INTRODUCTION

Organ transplantation is one of the success stories of the second part of the twentieth century. During the past 10 years results have steadily improved and patients undergoing kidney, liver, or heart transplantation can realistically anticipate approximate 80% and 70% one and 5-year survival, respectively. At the Oklahoma Transplantation Institute in Oklahoma City, for example, since 1987 almost 200 heart transplants have been performed with an overall one-year survival of 91% and a 7-year survival of 80% (1-3).

The major limiting factor to organ transplantation today is the increasing shortage of suitable donor organs. In the USA, approximately 45,000 people are listed for solid organ transplantation by UNOS, and yet less than 6000 cadaveric donors become available each year, from which approximately 20,000 donor organs are obtained. The discrepancy between the number of potential recipients and donor organs is increasing by approximately 10-15% annually (4). Patients on dialysis

awaiting kidney transplants are, therefore, waiting for longer and longer periods of time, and patients awaiting liver or heart transplantation may well die before a suitable donor becomes available.

One solution to this problem would be the use of animal organs - xenotransplantation (5,6). This field of research has been undergoing intensive and increasing study during the past few years, and some encouraging progress is being made.

3. CONCORDANT AND DISCORDANT XENOTRANSPLANTATION

3.1. Definitions

Xenotransplantation refers to the transplantation of organs or tissues from an animal of one species into another species. With regard to humans, it clearly refers to the use of a donor other than a human. The terms concordant and discordant xenografting are used loosely to refer respectively to transplantation between closely-related animal species (*e.g.* baboon-to-human) and between distantly-related species (*e.g.* pig-to-human) (7).

With regard to the histopathology of the rejection that takes place, we should probably confine our terms to (i) cellular, (iii) antibody-mediated (denoting vascular or humoral), and (iii) mixed rejection (8). Antibody-mediated rejection may be hyperacute (in that it occurs within minutes or a few hours after transplantation) or delayed, occurring some days or even weeks after transplantation.

3.2. Pathogenesis of graft rejection

In general, pre-existing antibodies are not present in humans in high titers against closely-related species (*e.g.* baboon), but can develop or rapidly increase during the first few days after concordant xenotransplantation. Rejection generally occurs in an accelerated fashion (when compared to that of an allograft) within a few days, and can be of a humoral, cellular, or mixed nature (8).

Significant differences in the rejection process occur in different species combinations and different transplanted organs. For example, cynomolgus monkey hearts transplanted into baboons would appear to be rejected primarily by a cellular mechanism (9,10), not unlike after allografting, whereas African green monkey hearts transplanted into baboons are more likely to be rejected by a humoral (or mixed humoral and cellular) mechanism (11,12). African green monkey livers transplanted into baboons, however, have been reported to be rejected primarily by a cellular mechanism (13).

The presence in humans of relatively high titers of natural pre-formed antibodies against discordant donor species (*e.g.* pig) leads to immediate hyperacute rejection (HAR) (as may occur when allografting is carried out in a sensitized recipient). The HAR is initiated by the interaction of the antibodies with antigens on the vascular endothelium of the donor organ, resulting in activation of the classical pathway of complement (14) and vascular endothelial cell activation and lysis. In some species combinations, the alternative pathway of complement activation is believed to play a role (15), and evidence has been put forward to suggest that in humans this may be due to dimeric IgA binding to the pig vascular endothelium (16).

If HAR can be avoided (*e.g.* by depletion of complement by cobra venom factor (17,18)), current evidence is that a delayed form of rejection occurs (often termed 'delayed xenograft rejection' (19)), which leads to more gradual graft failure. The exact mechanism of this delayed xenograft rejection remains uncertain, but appears to be antibody-mediated but complement-independent (19). There is increasing evidence that natural killer cells and macrophages may play significant roles (20).

3.3. Histopathology

The classical histopathologic picture of HAR consists of disruption of the vascular endothelium, with massive interstitial edema and hemorrhage (8) (Figure 1). Intravascular fibrin thrombi are frequently present, and platelet thrombi can be observed. This picture can, however, be considerably attenuated even when early graft failure has occurred.

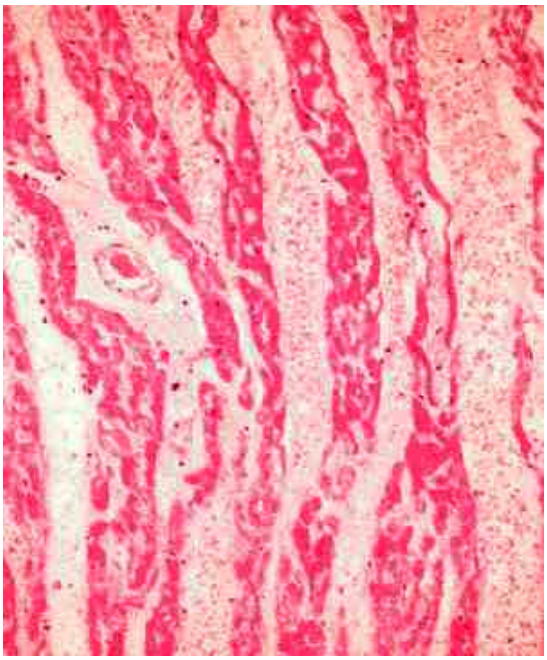

Figure 1: Photomicrograph of donor pig myocardium following xenotransplantation into a non-immunosuppressed recipient baboon. The donor heart ceased functioning after 4 hours, and histologically shows florid hyperacute rejection with severe interstitial hemorrhage, vascular thrombi, and myocyte necrosis. (Hematoxylin and eosin, x 150)

Table 1: Carbohydrate antigens which bound significant levels of human anti-pig heart or human anti-pig kidney antibodies.

ANTIGEN GROUP	TRIVIAL NAME	CHEMICAL STRUCTURE	NUMBER OF ANTI-PIG SAMPLES WITH STRONG BINDING (OD ³ 0.5)			
			Anti-pig Kidney		Anti-pig Heart	
			Human O plasma	Human AB plasma	Human O plasma	Human AB plasma
B-like	Linear B type 2	αGal(1→3)ßGal(1→4)ßGlcNAc-R	4/4	4/4	1/2	2/2
	Linear B type 6	αGal(1→3)ßGal(1→4)ßGlc-R	4/4	4/4	1/2	2/2
	B disaccharide	αGal(1→3)ßGal-R	4/4	4/4	1/2	2/2
	α-D-Galactoside	αGal-R	3/4	4/4	ND	ND
B	B type 4	αGal(1→3)ßGal(1→3)ßGalNAc-R³(1→2)αFuc	3/4	0/4	0/2	0/2
	B type 5	αGal(1→3)ßGal(1→3)ßGal-R³(1→2)αFuc	3/4	0/4	1/2	0/2
A-like	A disaccharide	αGalNAc(1→3)ßGal-R	2/4	0/4	1/2	2/2
	Linear A type 6	αGalNAc(1→3)ßGal(1→4)ßGlc-R	2/4	0/4	0/2	1/2
	Forssman disaccharide	αGalNAc(1→3)ßGalNAc-R	2/4	0/4	1/2	2/2
	Forssman trisaccharide	αGalNAc(1→3)ßGalNAc(1→3) αGal-R	3/4	0/4	1/2	2/2
A	A trisaccharide	αGalNAc(1→3)ßGal-R³(1→2) αFuc	3/4	0/4	0/2	0/2
	A type 4	αGalNAc(1→3)ßGal(1→3)ßGal NAc-R³(1→2)αFuc	2/4	0/4	1/2	0/2
	A type 5	αGalNAc(1→3)ßGal(1→3)ßGal-R³(1→2)αFuc	2/4	0/4	1/2	0/2
	A type 6	αGalNAc(1→3)ßGal(1→4)ßGlc-R³(1→2)αFuc	2/4	0/4	1/2	0/2
P	P1	αGal(1→4)ßGal-R	3/4	0/4	1/2	2/2
	Paragloboside	ßGal(1→4)ßGlcNAc(1→3)ßGal(1→4)ßGlc-R	0/4	0/4	1/2	0/2
ßGlcNAc-containing	N-Acetyl-ß-D-glucosaminide	ßGlcNAc-R	2/4	3/4	1/2	2/2
		ßGlcNAc(1→4)ßGlcNAc-R	0/4	1/4	1/2	2/2
Rhamnose containing	α-L-Rhamnose	α-L-Rha-R	0/4	2/4	1/2	2/2
		α-L-Rha(1→3)ßGlcNAc(1→2)α-L-Rha-R	2/4	2/4	1/2	2/2
Other		αGalNAc(1→2)ßGal-R	1/4	1/4	1/2	2/2
		αGalNAc(1→4)ßGal-R	1/4	0/4	1/2	1/2
		ßGalNAc(1→2)ßGal-R	0/4	2/4	1/2	2/2
		ßGal(1→3)ßGalNAc-R	0/4	1/4	1/2	0/2
		αGlc(1→2)ßGal-R	1/4	0/4	1/2	1/2
		ßGlc(1→2)ßGal-R	1/4	0/4	1/2	1/2
		ßGlc(1→2)αMan-R	1/4	1/4	1/2	1/2
		αMan(1→6)αMan-R	0/4	1/4	1/2	2/2

R=O-(CH2)8-CO-NH-bovine serum albumin, Gal=galactose, Fuc=fucose, Rha=rhamnose, GlcNAc=N acetylglucosamine, GalNAc=N acetylgalactosamine, Man=mannose, ND=not done.

(Modified from DKC Cooper, *et al.* (24))

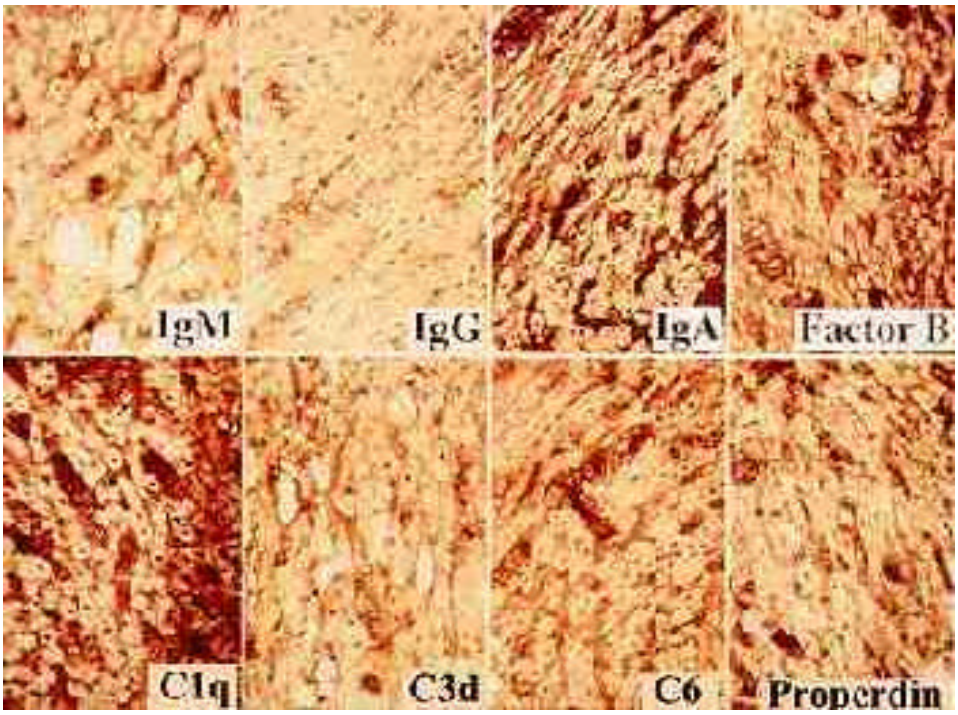

Figure 2: Immunoperoxidase labeling of a pig-to-baboon cardiac xenograft that was rejected hyperacutely. There is endothelial deposition of IgM, IgG and IgA. The graft also shows endothelial deposition of components of the classical (C1q) and alternate (Factor B, properdin) pathways of complement activation, along with C3d and terminal pathway components (*e.g.* C6). (Courtesy W.W. Hancock)

Immunofluorescence studies demonstrate IgM, IgG, IgA and complement deposition on the vascular endothelium (21, and Kobayashi, T., *et al.*,submitted for publication) (Figure 2). The histo-pathological features of delayed xenograft rejection vary little from those seen in HAR, although immunohisto-logical studies reveal the presence of cytokines and various cells (21, and Kobayashi, T., *et al.*, submitted for publication).

3.4. Anti-pig antibodies

Current evidence is that all (or most) human anti-pig antibodies are directed against αgalactosyl (αGal) epitopes, specifically with a terminal Galα1-3Gal structure, on the surface of pig vascular endothelium (22-28) (Tables 1 and 2 and Figure 3). These anti-αGal antibodies are also found in apes and Old World monkeys, but not in lower primates (*e.g.* New World monkeys) or non-primate mammals (including the pig), which, in contrast, express the αGal antigen (29). Following the transplantation of a pig organ into a human or baboon, or the extracorporeal perfusion of human blood through a pig organ, there is a marked increase in the titer of anti-αGal antibody, increasing by <60-fold over a period of days or weeks (30-32).

Humans are believed to develop anti-αGal antibodies during the first few weeks of life through exposure to certain microorganisms that colonize the gastrointestinal tract and which also express αGal structures on their cell membranes (33). At birth, anti-αGal IgG can frequently be detected in the plasma, presumably passively transferred from the mother, but not IgM (34). As it is predominantly IgM binding that initiates HAR, a pig organ transplanted into a neonatal baboon is not rejected hyperacutely, but does undergo delayed xenograft rejection over the next few days (34).

TABLE 2: Structure of the main carbohydrate epitopes exposed at the surface of human and porcine vascular endothelium.

HUMAN	PIG
ßGal1-4ßGlcNAca-R*	ßGal1-4ßGlcNAca-R
ABH-ßGal1-4ßGlcNAca-R	αGal1-3ßGal1-4ßGlcNAca-R
αNeuAc2-3ßGal1-4ßGalNAca-R	αNeuAc2-3ßGal1-4ßGlcNAca-R

*R are glycolipid or glycoprotein carrier molecules anchored in the cell membrane. Only the epitopes underlined are different between the two species. (From R Oriol,., *et al.* (25))

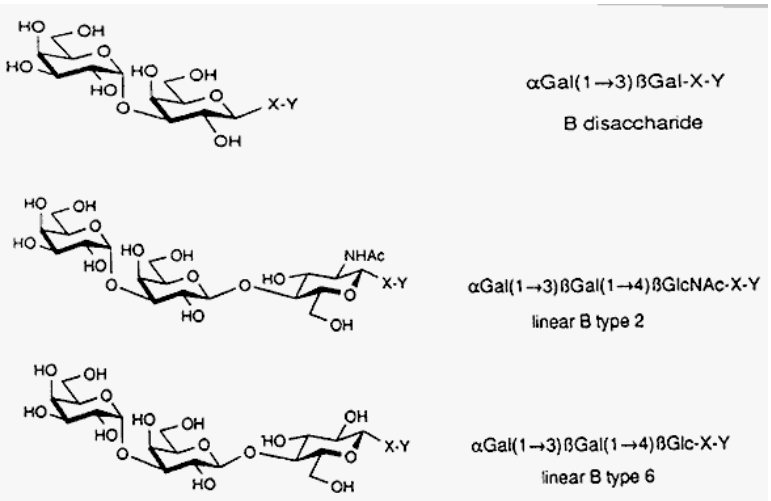

Figure 3: Three of the major carbohydrate structures that bind human antibodies eluted from pig heart, kidney and red blood cell stroma - αGal disaccharide (above), αGal trisaccharide type 2 (center), and αGal trisaccharide type 6 (below). X-Y = $(CH_2)_8COOHCH_3$.

4. EXPERIMENTAL METHODS OF PROLONGING SURVIVAL OF CONCORDANT XENOGRAFTS

Most work in this area has been directed at the use of pharmacologic immunosuppressive agents and, to a lesser extent, total lymphoid irradiation (TLI). In early studies, heavy pharmacologic immunosuppressive therapy was shown to extend African green monkey heterotopic heart survival in baboons for several weeks, though there was a relatively high morbidity and mortality from infection in the recipient animals (12). African green monkey livers have been demonstrated to function for >12 months in baboons (13,35,36), despite a relatively high incidence of cellular rejection episodes.

TLI has similarly been shown to extend kidney or heart survival in concordant species combinations, particularly in association with pharmacologic agents (37-41).

Survival of orthotopically transplanted concordant heart xenografts was investigated by Kawauchi *et al.* (42), who performed the procedure using rhesus monkeys as donors and juvenile baboons as recipients. In six control experiments in which no immunosuppressive therapy was given, the recipients survived for a mean of eight days and all died of classic cellular rejection. In five baboons that underwent splenectomy and were treated with perioperative antilymphocyte globulin and long-term maintenance FK506 (tacrolimus) (with intravenous methotrexate, methylprednisolone, or both as rescue therapy in cases of severe rejection), mean survival was extended to 48 days. Two baboons died from rejection and three from cytomegalovirus infection.

Seven additional baboons received the same immunosuppression but with an added intravenous (i.v.) dose of methotrexate given twice weekly. Mean survival in this group was 127 days, and one baboon was still alive after 286 days. Four died from infection, one from pulmonary embolism, and one from renal failure. Only two of the baboons that died showed mild rejection at autopsy.

Although the authors concluded that FK506 coupled with low-dose maintenance methotrexate and splenectomy can produce prolonged host survival in this model, the quantity of immunosuppression given was clearly considerable and resulted in death from infection in a large number of recipient animals. If this degree of immunosuppression were required to provide long-term survival of concordant xenografts in humans, it would likely lead to a high incidence of recipient morbidity or mortality from infection.

With the current immunosuppressive agents available to us, it seems unlikely that even concordant donor organs will survive for very prolonged periods of time (years) in human recipients. If standard immunosuppressive therapy is given, there will be a risk of organ failure through recurrent or severe acute rejection and/or graft arteriosclerosis (chronic rejection). Function for some months is likely to be achieved, but the amount of immunosuppressive therapy required is likely to be excessive and will almost certainly lead to infectious complications.

Some of the newer immunosuppressive agents may well prove to be more efficacious in prolonging concordant xenograft survival. In particular, leflunomide (43,44), brequinar sodium (45,46), and 15-deoxyspergualin (47-51) have shown encouraging results in some experimental models, although there remain few data on studies in large animals, particularly in primates. There is some prospect, however, that a combination of

the immunosuppressive agents that will become available to us within the next few years may well enable concordant xenotransplantation to be carried out successfully, particularly as a bridge to allotransplantation in cases of cardiac or liver failure.

Concordant xenotransplantation in humans will be limited, however, by the relative paucity of the number of suitable donor animals that will become available and, in particular, on the size of such animals. The baboon does not grow to a size sufficient to provide organs such as hearts for adult humans, although there may be a role for it in bridging infants and children to cardiac allotransplantation. Baboons are known to carry certain infectious agents, particularly viruses, that may be hazardous if transferred to humans (52-55). In addition, there will likely be a significant public objection to the use of non-human primates in large numbers for purposes of transplantation. Increasingly, therefore, the attention of those interested in this field has been directed towards discordant xeno-transplantation.

5. EXPERIMENTAL METHODS OF PROLONGING SURVIVAL OF DISCORDANT XENOGRAFTS

Most attention has to-date been paid towards overcoming HAR for, if this cannot be achieved, then there seems little purpose in directing efforts to overcoming the delayed xenograft rejection (and cellular rejection) that occurs subsequently. Work in this field can loosely be divided into 4 main approaches (Table 3).

(i) Depletion in the recipient of anti-pig antibodies or inhibition of their attachment to graft antigens.

(ii) Depletion or inhibition of complement in the recipient.

(iii) The development of immunological tolerance in the recipient to donor tissues by the creation of mixed chimerism.

(iv) Genetic engineering of a donor pig whose organs are protected from the antibody-mediated complement activation that would follow transplantation into a human.

The currently available pharmacologic immunosuppressive agents are totally ineffective in preventing HAR, but have been shown to play a role in reducing the rapidity of delayed xenograft rejection (17,18). There is no evidence to-date, however, that they can totally prevent delayed vascular rejection. Clearly, until this hurdle has been overcome, their role in the prevention of other cellular responses that are likely to follow remains uncertain.

5.1. Anti-pig antibody depletion or inhibition

The potential recipient can be depleted of all antibodies by plasma exchange (56), or of some antibodies by immunoadsorption techniques using immunoaffinity columns consisting of, for example, staphyloccocal protein A (17,57,58). However, these techniques deplete the patient of antibodies that may be important in protecting against infection. A preferable technique is to utilize highly specific extracorporeal immunoaffinity columns where only those anti-pig antibodies that are detrimental to the transplant will be depleted (22-24). This can be achieved by utilizing an extracorporeal immunoaffinity column of an αGal oligosaccharide (59-61, and Taniguchi, S., *et al.*, submitted for publication).

Table 3: Methods of prevention of hyperacute rejection in discordant xenotransplantation.

Directed at the Recipient
• Depletion or inhibition of anti-pig antibody
• Depletion or inhibition of complement
• Development of immunological tolerance to donor tissues (mixed chimerism)
Directed at the Donor*
• Replacement of aGal epitopes by expression of another carbohydrate structure
• Expression of human complement inhibiting proteins

***By genetic engineering techniques or possibly by gene therapy.

Such a technique has been shown to be successful clinically with regard to depletion of anti-A or anti-B histo-blood group antibodies in patients receiving ABO-incompatible organs (62) or bone marrow (63,64) allografts, where the pattern of HAR that can occur in the unmodified recipient is almost identical to that seen in discordant xenotransplantation. There is increasing evidence that it will also be successful with regard to depletion of anti-αGal antibodies.

Anti-αGal or anti-A or anti-B antibodies clearly return once the course of extracorporeal immunoadsorption is discontinued, although concomitant pharmacologic immunosuppressive therapy (and possibly splenectomy) prevents significant antibody rebound and maintains a low level of antibody. In the case of organ allografting across the ABO barrier, the return of antibody directed against target epitopes on the donor organ does not result in rejection of the organ. The mechanism by which this phenomenon (known as accommodation (65,66)) occurs remains unknown. It is not yet certain that accommodation will take place after discordant xenotransplantation in temporarily antibody-depleted recipients.

An alternative approach would be to carry out what has been termed "specific intravenous carbohydrate therapy," in which synthetic or natural αGal oligosaccharides are infused continuously into the recipient circulation (23,67-69). The oligosaccharides are bound by the anti-αGal antibodies in the blood, causing

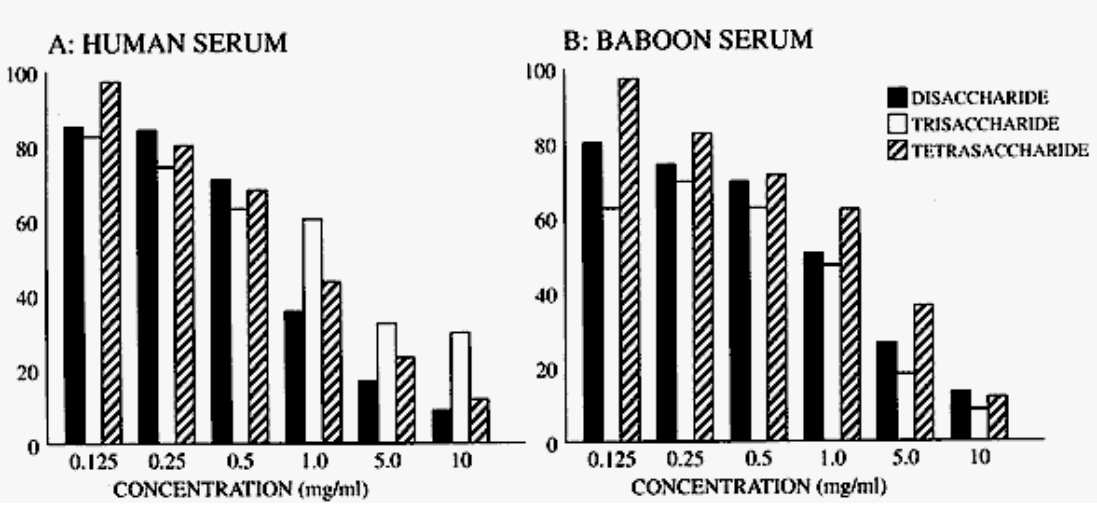

Figure 4: Reduction in cytotoxicity of (A) human and (B) baboon serum on PK15 cells after incubation of serum with increasing concentrations of α-galactosyl oligosaccharides (Dextra, Reading, UK) B-disaccharide = Galα1-3Gal; B-trisaccharide = Galα1-3Galß1-4Gal; B-tetrasaccharide = Galα1-3Galß1-4Galα1-3Gal. (From Neethling, F.A., *et al.* (59))

"neutralization" of the antibodies so that they are no longer free to attack the pig organ when it is transplanted.

Once again, this has been demonstrated to be a successful experimental approach with regard to inhibition of anti-A or anti-B antibodies. There is now a considerable amount of *in vitro* (Figure 4) (59) and a little *in vivo* (Figure 5) (60,61, and Taniguchi, S., *et al.*,submitted for publication) evidence that this approach may also be successful with regard to anti-αGal antibodies.

The major limiting factor of the above approaches at the present time has been the difficulty and expense of synthesizing these oligosaccharides in the large quantities required, particularly if they are to be infused i.v., when very large quantities are required. This problem is likely to be overcome in the near future by the use of enzymatic methods to produce the relevant oligosaccharides in kilogram quantities. The αGal oligosaccharide must be of the Galα1-3Gal configuration at its reducing end, and can be a di-, tri-, tetra-, or a pentasaccharide. There is evidence that the structure of the non-reducing end also plays a role (though less significant) in the efficiency of the oligosaccharide to inhibit antibody (Table 4) (70).

A search for a cheap source of aGal oligosaccharides has revealed that pig stomach mucin, which is readily available commercially, contains a subfraction that is highly efficient in inhibiting human and baboon anti-αGal antibody lysis of pig cells in culture (71,72).

An alternative to the use of αGal oligosaccharides, either in immunoaffinity columns or as an i.v. infusate, is the anti-idiotypic antibody. Koren *et al.* (73, 74) have produced anti-idiotypic antibodies in mice by the injection into the mouse of human anti-pig antibody (eluted from pig organs after repeated perfusion with human plasma). Several of these anti-idiotypic antibodies, when incubated with human serum, have been demonstrated to have a major inhibitory effect on serum cytotoxicity towards pig PK15 cells *in vitro*. Furthermore, when infused i.v. in combinations of two into baboons, serum cytotoxicity has again been markedly reduced (from 100% to approximately 10%).

5.2. Complement depletion or inhibition

Purified cobra venom factor (CVF) has been shown to be extremely effective in depleting complement and can clearly protect a discordant organ from HAR (17,18,58). However, even when the complement level is unmeasurable by standard laboratory tests, histopathological features of delayed xenograft rejection begin to develop within 2-3 days and lead to graft failure within a relatively short period of time (<1 week) (18). The addition of concomitant pharmacologic immunosuppressive therapy, presumably by suppressing both B and T cell activity, delays rejection further, but mixed vascular and cellular rejection is seen within days with the longest survival of a pig organ in a nonhuman primate to-date being 27 days (18).

Soluble complement receptor type I (sCR1) has also had success in prolonging discordant xenograft function (75-78). Human complement receptor 1 is a single-chain cell-surface glycoprotein found on erythrocytes, some T lymphocytes, all mature B lymphocytes, neutrophils, eosinophils, basophils, monocytes/macrophages, and certain other cells (79). It is also found circulating as a soluble form in plasma at low concentrations. The interaction of complement receptor 1 with some fractions of the complement cascade regulates complement activation through its convertase decay

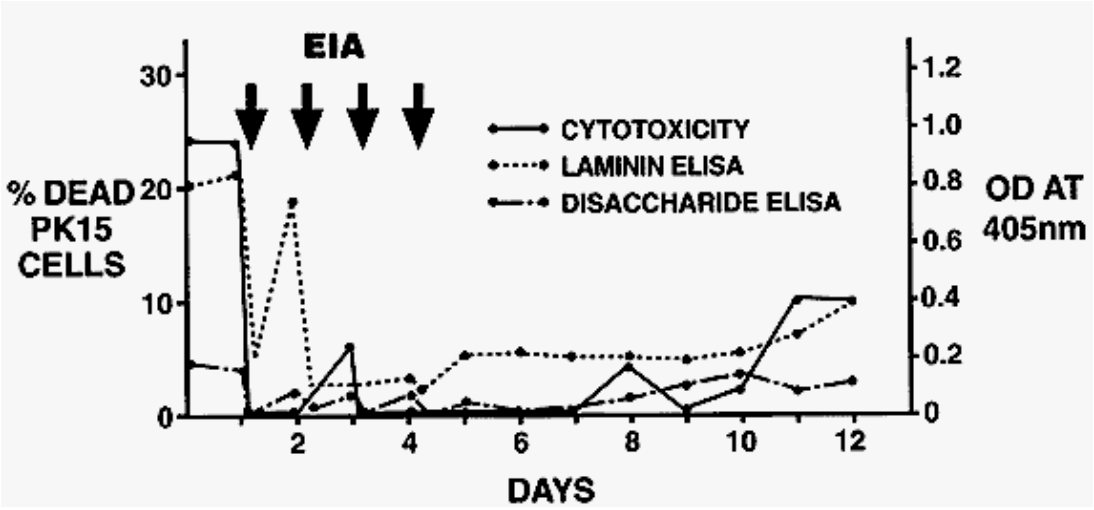

Figure 5: Serum cytotoxicity to PK15 cells and anti-αGal antibody levels (measured by mouse laminin ELISA and αGal disaccharide ELISA) in a baboon that underwent a total of 4 extracorporeal immunoadsorptions using an immunoaffinity column of αGal disaccharide. Cytotoxicity to the serum was immediately depleted after the first EIA and remained at extremely low or insignificant levels for approximately 10 days. (From Taniguchi, S., *et al.*, submitted for publication)

Table 4: Oligosaccharide concentrations

Oligosaccharide*	**Concentration (μM) in serum**	
	Human	**Baboon**
1. *Fucα1-2Galß1-R	>10000	>10000
2. Galß1-R	>10000	>10000
3. Galα1-2Galß1-R'	7000	>10000
4. Galα1-3Gal	386(±149)[a]	301(±44)[e]
5. Galα1-3Galß1-4Gal	163(±73)	141(±60)
6. Galα1-3Galß1-4Galα1-3Gal	54(±31)	119(±30)
7. Galα1-3Galß1-4GlcNAc	27(±11)	31(±4)

*Underlined type indicates structural differences of the oligosaccharide from the major pig vascular endothelium glycolipid Galα1-3Galß1-4GlcNAcß1-3Galß1-4Glcß1-Cer. R represents 1-3 or 1-4 linkages to Gal or to GlcNAc; R' is $-O(CH_2)_3NHCOCH_3$. The results of the strong inhibitors (a-h) are expressed as mean ± SD (n=3). Statistical significance: a vs c (t=3.1); a vs d (t=3.9); b vs d (t=3.1); e vs h (t=9.7); f vs h (t=3.1); g vs h (t=5.4), all with P<0.02. The other comparisons did not reach the P=0.05 level of significance.(From Neethling, F.A., *et al.* (70))

accelerating activity and its factor 1 cofactor activity (79-81). Fearon and colleagues constructed a soluble form of complement receptor 1 which lacked the transmembrane and cytoplasmic protein domains (81). This sCR1 retains all the known activities of the native cell surface receptor, and has been demonstrated to be a potent and selective inhibitor of both the classical and alternative complement pathways. Discordant xenografts have survived for over three weeks when protected by sCR1 (82).

It would seem, however, that complement depletion or inhibition alone, although valuable therapeutic approaches to assist in overcoming HAR, will not be sufficient to prolong discordant xenograft survival indefinitely.

5.3. The development of tolerance to donor species by mixed chimerism

Donor species-specific tolerance would clearly be desirable and may indeed prove essential if late rejection of a discordant xenograft proves to be significantly more severe than that of an allograft. Important studies have been carried out over a number of years in experimental animals by two groups, namely those headed by Myburgh at the University of the Witwatersrand in Johannesburg (83-85) and by Sachs, formerly at the National Institutes of Health in Bethesda and more recently at Harvard Medical School (86-93).

The induction of donor specific tolerance would clearly eliminate the development of acute or chronic rejection. The elimination of chronic rejection (*e.g.* graft atherosclerosis or bronchiolitis obliterans) is possibly even more important than that of acute rejection as there is no effective treatment for chronic rejection even in allografts. If tolerance could be achieved, pharmacologic immunosuppressive therapy would not be necessary and therefore the accompanying risks of opportunistic infection, malignancy, and drug toxicity would be avoided.

Sykes and Sachs (93) have pointed out that the tolerance approach may be well suited for xenotransplantation since animal donors are available electively (and not under emergency conditions as are cadaveric human donors) allowing for the timing of

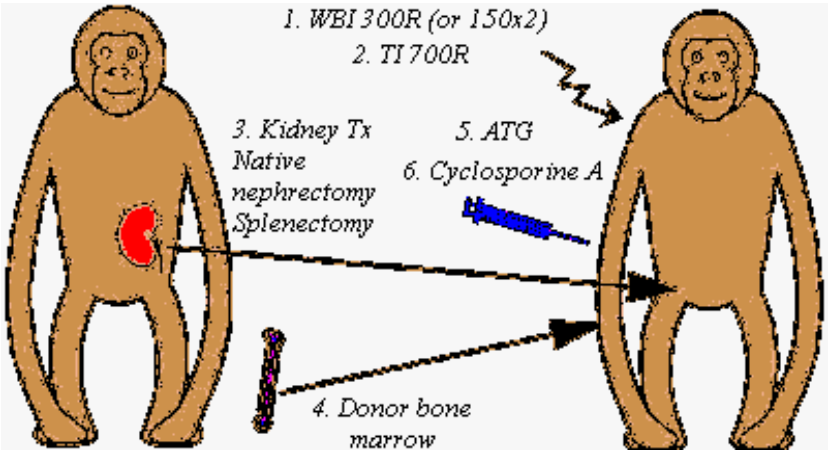

Figure 6: Schematic representation of protocol for non-myeloablative preparative regimen to induce tolerance between full MHC haplotype-mismatched cynomolgus monkeys. In attempting to induce transplantation tolerance across the discordant xenogeneic barrier pig-to-cynomolgus monkey through establishment of mixed chimerism, extracorporeal immunoadsorption of monkey blood through a pig liver or immunoaffinity column of a Galα1-3Gal oligosaccharide is performed prior to the pig kidney transplant in an effort to deplete anti-pig antibody. Additional pharmacologic immunosuppressive therapy, *e.g.* with 15-deoxyspergualin, has been used to inhibit B cell activity and therefore reduce the rate of return of antibody. (From Sykes, M. and Sachs, D.H. (93))

tolerance induction and transplantation to be elective. Tolerance-inducing cell populations (*e.g.* bone marrow) can be obtained from the donor, the recipient can undergo the procedure to induce a tolerant immune system, and the organ graft from the same donor can be inserted at the optimum time. In addition, the potential for generating fully inbred xenograft donors (*e.g.* miniature swine) provides the possibility of using an unlimited source of genetically homogeneous tissue whenever it is required for maintenance of the tolerant state. Xenogeneic donors could be modified using genetic engineering or gene therapy techniques to facilitate induction of tolerance to xenoantigens.

Two approaches are being investigated by the Harvard group (93), namely (i) the use of xenogeneic hematopoietic cell transplantation to induce a state of mixed chimerism, and (ii) thymectomy followed by replacement with a xenogeneic donor thymus after depletion of the preexisting peripheral T cell repertoire. In this brief review, only the mixed chimerism approach will be discussed.

After much preliminary work in rodents, the Sachs group has investigated the development of tolerance to allografts and xenografts in nonhuman primates. The basic protocol (Figure 6) consists of the nonhuman primate receiving 3.0 Gy of whole body irradiation (WBI), 7.0 Gy of thymic irradiation, and horse anti-human ATG preoperatively. Bilateral nephrectomy, splenectomy, orthotopic kidney transplantation, and donor bone marrow administration are all performed on day 0. In order to supplement suppression of mature T cells by ATG, treatment with cyclosporine intramuscularly is begun on day 1 and continued for 4 weeks, but then no further immunosuppression is administered.

Clear evidence for chimerism amongst lymphoid, myeloid, and monocytic subpopulations, was generally detected first on about day 8, persisting until about day 30. Thereafter the levels of detectable chimerism decreased progressively. However, in recipient animals given allografts and concordant xenografts, transplantation tolerance was induced, as assessed by MLR assays, by monitoring of kidney transplant function, and in one case by acceptance of a full thickness graft of frozen skin from the kidney donor.

More recent studies have attempted to extend this non-myeloablative regimen for production of mixed chimerism in the discordant pig-to-primate combination (92,93). The major addition to previous protocols is the need to remove natural antibodies from the recipient circulation in order to avoid HAR. This has been attempted by extracorporeal perfusion of the monkey's blood either through an isolated pig liver or through specific synthetic oligosaccharide columns and has been carried out immediately prior to kidney transplantation.

Using this regimen, pig kidney grafts have functioned normally for <15 days in cynomolgus monkeys, but have failed from a vascular form of rejection. In addition, there has been only transient evidence for pig cell chimerism, with a low level of pig cells (1-5%) in the peripheral blood.

5.4 The genetically engineered pig

5.4.1. Expression of human complement-inhibiting proteins

Most advances in this field have come from

efforts to genetically engineer a pig that expresses one or more of the human complement-inhibiting proteins. Complement-inhibiting proteins are believed, under most circumstances, to block autologous complement but not that of other species (94,95). For example, pig organs express complement-inhibiting proteins that block pig complement, but do not adequately block human complement. It is therefore believed that the development of a pig that expresses human complement-inhibiting proteins on its vascular endothelium will be successful in blocking human complement.

The human complement-inhibiting proteins include CD46 (membrane cofactor protein, MCP), CD55 (decay accelerating factor, DAF), and CD59 (homologous restriction factor). Pigs have been bred that express one or more of these proteins (96-101), but few data are yet available on their efficacy at preventing HAR. Survival of pig hearts expressing MCP and DAF was extended from a few minutes to 30 hours in one baboon. The most encouraging results achieved to-date have been by the Cambridge, UK, group of White and his colleagues (101), who have reported heterotopic pig heart survival for <60 days in one cynomolgus monkey. This model of pig-to-cynomolgus monkey, however, is unusual in that some of the control (non-transgenic) pig hearts survived several days, suggesting that HAR is not uniform in this combination. Investigations in this field are progressing rapidly.

5.4.2. αGal 'Knockout'

The second approach with regard to a genetically engineered pig would be to produce a pig that is deficient in αGal epitopes, thus leaving no target for human anti-αGal antibodies (102). In the pig, Galα1-3Gal is produced by the enzyme α1,3galactosyltransferase (α1,3GT), which is encoded by a single gene (29). If this gene could be "knocked out" by a technique such as homologous recombination, then an αGal-deficient pig would be produced. The only hitherto discovered difference between pigs and humans with regard to the oligosaccharides expressed on the vascular endothelium is the presence of αGal in the pig where ABH oligosaccharide is expressed in the human (25) (Table 2). Whether an αGal-depleted pig would be a fully viable, healthy pig remains uncertain, but the fact that there are some human subjects who are depleted of ABH antigen (the so-called "Bombay" histo-blood type) who appear to be clinically well in all respects, would suggest that αGal-depleted pigs will similarly be healthy.

The "knockout" technique, which requires the manipulation of stem cells, is not yet possible in the pig. Mice, however, have been bred which do not express αGal epitopes (103,104). One strain of these mice have certain physical defects in the form of the early development of cataracts (103). *In vitro* and *in vivo* studies, however, suggest that the absence of αGal exposes the presence of underlying "cryptic" oligosaccharide epitopes against which humans also have antibodies. This approach would not appear so promising as originally hoped.

Table 5: Fucosylation or sialylation of N-acetyl-lactosamine (ßGal1-4ßGlcNAc-R) can impair the formation of terminal non-reducing αGal epitopes

Chemical Structure	Epitope Name
αFuc1-2ßGal1-4ßGlcNAc-R*	H
ßGal1-4ßGlcNAc-R1-3αFuc	Lewis
αNeuAc2-3ßGal1-4ßGlcNAc-R1-3αFuc	Sialyl-Lewis
αNeuAc2-3ßGal1-4ßGlcNAc-R	Sialyl-3-lactosamine
αNeuAc2-6ßGal1-4ßGlcNAc-R	Sialyl-6-lactosamine

*R are glycolipid or glycoprotein carrier molecules anchored in the cell membrane. The presence of fucose or sialic acid represents a steric hindrance for the enzyme activity of the pig α(1,3) galactosyltransferase. Added fucose or sialic acid are underlined. (From DKC Cooper, *et al.* (26))

5.4.3. Competitive glycosylation

One alternative approach would be to genetically engineer a pig with an abundance of another oligosaccharide epitope that would "mask" the αGal epitope. Suggested candidates have been sialic acid or the H histo-blood group antigen (Table 5) (26,102). This method, involving the microinjection of a gene to express the required oligosaccharide, is possible in the pig. What percentage of αGal expression needs to be "masked" before HAR is prevented remains uncertain, but it seems likely that it will be virtually 100%.

Good progress in this field has been made by Sandrin *et al.* (105,106) who have demonstrated *in vitro* that competition between α1,2 fucosyltransferase (H transferase) and α1,3GT takes place for the substrate N-acetyllactosamine (Figure 7). H transferase is significantly more successful and the H epitope predominates, reducing the presence of αGal to approximately 5% of its original expression.

One interesting point is that pigs do, in fact, have the gene for H transferase and express H oligosaccharide epitopes, not on vascular endothelium but in certain other tissues (25). It is therefore essential to ensure that the H transferase produced as a result of the introduction of H cDNA functions at the correct site, and this may prove to be less easy than is immediately obvious.

Unless H epitopes replace the αGal epitopes completely, the number of αGal epitopes remaining on the vascular endothelium would still make such a pig organ susceptible to HAR. The ultimate solution, therefore, may be to combine expression of H transferase with that of αgalactosidase (107) (Figure 7). The remaining epitopes expressing αGal will be depleted of the αGal by αgalactosidase, rendering the N-acetyllactosamine again

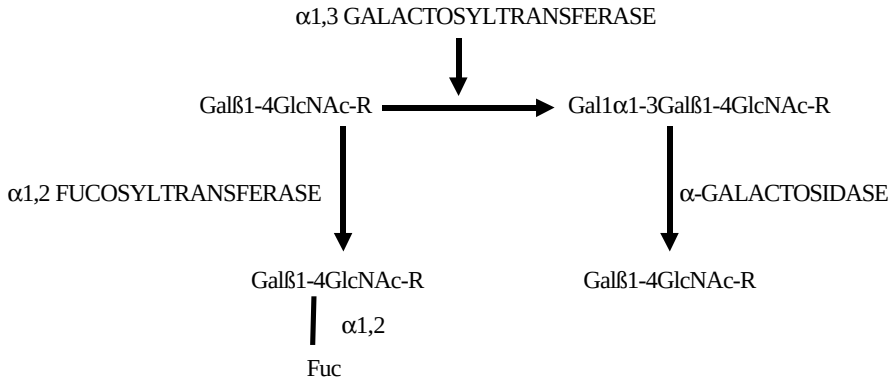

Figure 7: Biosynthetic pathway for synthesis of Galα1-3Gal. The α1,3 galactosyltransferase enzyme adds galactose to N-acetyllactosamine (Galß1-4GlcNAc) to generate Galα1-3Gal. The same substrate can be utilized by transgenically-introduced α1,2 fucosyltransferase to produce the H histo-blood group epitope. Galα1-3Gal can also be eliminated by the introduction of α-galactosidase, which enables the N-acetyllactosamine substrate to be available again for further fucosylation. (Modified from Sandrin, M.S., *et al.* (106))

available for the H transferase.

At the present time, this approach of modifying the donor organ by deletion of αGal expression, together with the expression of complement-inhibiting proteins (perhaps in addition to providing some therapy to the recipient) would appear to be the most promising method of overcoming HAR and of being able to apply discordant xenotransplantation clinically.

6. DISCUSSION

Methods that allow successful discordant xenotransplantation will clearly open up new areas of surgical therapy. Patients with native organ failure who are in need of a transplant will be able to undergo the procedure electively or immediately the need arises. They will no longer be condemned to wait anxiously in precarious health for weeks, months or even years before ultimately undergoing transplantation as an emergency procedure at a less-than-optimal time of the day or night. Patients with borderline contraindications to allotransplantation will be given the opportunity of xenotransplantation as there will no longer be a restriction on the number of donor organs. Transplantation will become a common procedure in countries such as Japan where to-date the absence of brain death laws has prevented allografting except from living-related donors. Diabetic patients may receive pig pancreatic islet cell transplants (108), negating the need for daily porcine insulin injections. The ethical problem of whether retransplantation should be offered to a patient will be overcome by the abundance of donor organs.

There will therefore be a great expansion in organ transplantation worldwide (4) and it is likely that both patients and physicians will not wish to persist with inadequate medical therapy, including dialysis, if successful organ xenotransplantation is readily available.

Will the public accept an organ from a pig? (109). Pig heart valves have been utilized for many years and have become acceptable to the public in general, including those of the Jewish and Muslim faiths. Porcine insulin is used in millions of diabetics worldwide. The results of a survey carried out in the USA in 1993 indicated that 84% of those questioned would accept an allograft if they needed one and no fewer than 51% would accept a xenograft if no allograft was available (110). As the people surveyed were presumably healthy individuals who did not anticipate the need for transplantation even in the remote future, the 51% acceptance rate for a xenograft was surprisingly high. It is likely that if the survey had been confined to patients awaiting an organ transplant, particularly those on life-support in an intensive care unit, then the percentage who would have accepted a xenograft would have been very much higher.

In an interesting editorial, Chen and Michler (111) discussed the difficult question of when to initiate a clinical heart xenotransplantation program. They suggested (quoting the work of Fox and Swazey (112)), that three questions need to be answered, namely "(i) in the laboratory, what defines success of a sufficient level to warrant advancement to the clinical arena? (ii) under what clinical condition should this advancement proceed? and (iii) in the clinical arena, what defines success of a sufficient level to warrant further evaluation?" They do not provide conclusive answers to these questions but clearly believe that "the question that currently remains is not how, but rather when should heart xenotransplantation advance to the clinical arena."

Which of the methods and approaches briefly outlined in this review is most likely to be successful in allowing clinical organ xenotransplantation? The answer is probably a combination of techniques and/or agents, as is the case with allotransplantation today. It is unlikely that one single approach will be entirely successful. Those

with insight in this respect should be encouraged to heed the words of William Shakespeare: -

"If you can look into the seeds of time,
And say which grain will grow and which will not,
Then speak to me." (Macbeth)

Accepting the difficulty of predicting the future, it would seem that xenotransplantation offers us the first real opportunity for modifying the donor as opposed to the recipient. This opens up great possibilities, particularly in this era of rapidly developing techniques such as genetic engineering and gene transfer. The breeding of a pig with a vascular endothelial structure against which humans have no pre-formed antibodies (and are unlikely to develop new antibodies) will be a major advance, and seems possible in the near future. Similarly, the ability of the pig organ to block the destructive effect of human complement will help overcome HAR. If neither of these techniques alone, or in combination, is entirely successful in preventing antibody-mediated rejection, then the inhibition of known anti-αGal antibodies by the infusion of an αGal oligosaccharide may lead to accommodation.

Even when we have successfully overcome the antibody-mediated complement-dependent HAR and the antibody-dependent delayed vascular rejection, we will almost certainly face further problems from a cellular response, which is likely to be severe (113,114). However, several novel pharmacologic immunosuppressive agents are currently being evaluated that lend hope that this hurdle will also be successfully overcome (43-51). Looking even further into the future, it is hoped that immunological donor-specific tolerance can be achieved in the human host to transplanted pig organs, thus negating the need for long-term immunosuppressive therapy and minimizing the late complications of such therapy.

There will remain, however, several unknowns. Will the porcine organ function satisfactorily in the human environment? (109,115). Pig hearts have functioned successfully in the heterotopic position in nonhuman primates for several weeks (18,101), as have pig kidneys (56). It is likely that both of these organs will fulfill the functions required of them in the human host. It is much less likely that a transplanted pig liver will fulfill all of the roles expected of it. Will pig proteins, enzymes, and hormones carry out their tasks in the human? (115). It is inconceivable that the products of a pig liver will be completely interchangeable with those of a human liver, but here again, in time, genetic engineering of the donor pig may allow some of these functions to occur satisfactorily.

We already have clinical evidence, however, that *ex vivo* perfusion of pig livers by blood from human patients in fulminant hepatic failure can lead to some improvement in cerebral activity, and therefore at least temporary support by a pig liver is likely to be beneficial (116,117). One area of interest and speculation is, of course, that after orthotopic transplantation using a pig liver, the liver will produce pig complement. This should help to protect the transplanted organ from HAR, but what effect it will have on the remaining human organs in the body and on the body's defense mechanisms to infection remains unknown.

There are those with a cynical outlook who for many years have predicted that "the future of transplantation is xenotransplantation, and always will be!". Unfortunately, to-date they have been proved correct! In the final decade of this century, however, we at last appear to be making some real progress, and there are glimpses of light at the end of the tunnel of ignorance and failure. I prefer, therefore, the much more positive attitude of George Bernard Shaw's character in "Back to Methuselah" who says:

"You see things: and you say 'why?'
Always 'why?'
But I dream things that never were:
And I say 'why not?'"

Nevertheless, there will undoubtedly be many pitfalls and disappointments ahead. The future has probably best been summed up by Professor Roy Calne, the pioneering transplant surgeon, who recently stated: "Clinical xenotransplantation is just around the corner, but unfortunately it may be a very long corner."

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