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Review

Xenotransplantation: the importance of the Galα1,3Gal epitope in hyperacute vascular rejection

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Abstract

The transplantation of organs from other species into humans is considered to be a potential solution to the shortage of human donor organs. Organ transplantation from pig to human, however, results in hyperacute rejection, initiated by the binding of human natural antidonor antibody and complement. The major target antigen of this natural antibody is the terminal disaccharide Galα1,3Gal, which is synthesized by Galβ1,4GlcNAc α1,3-galactosyltransferase. Here we review our current knowledge of this key enzyme. A better understanding of structure, enzyme properties, and expression pattern of α1,3-galactosyltransferase has opened up several novel therapeutic approaches to prevent hyperacute vascular rejection. Cloning, and expression in vitro of the corresponding cDNA, has allowed to develop strategies to induce immune tolerance, and deplete or neutralize the natural xenoreactive antibody. Elucidation of the genomic structure has led to the production of transgenic animals that are lacking α1,3-galactosyltransferase activity. A detailed knowledge of the enzyme properties has formed the basis of approaches to modify donor organ glycosylation by intracellular competition. Study of the expression pattern of α1,3-galactosyltransferase has helped to understand the mechanism of hyperacute rejection in discordant xenotransplantation, and that of complement-mediated, natural immunity against interspecies transmission of retroviruses.

Keywords: Xenotransplantation; Hyperacute rejection; α1,3-Galactosyltransferase; Natural antibodies; Glycosylation

Contents

Abbreviations: α3GalT, UDP-Gal:Galβ1,4GlcNAc α1,3-galactosyltransferase (EC 2.4.1.87); α-gal glycotope, Galα1,3Gal-R; GSI-B4, *Griffonia simplicifolia* lectin-IB_4; HAR, hyperacute rejection

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1. Introduction

During the last 10 years, the number of organ transplantations has slowly increased. Nevertheless, in the United States the number of patients on the waiting list for operation has tripled over the same period of time (Nature 391 (1998) 325). This situation is due to the shortage of suitable human organs, resulting from an increased demand, and an insufficient supply. Theoretically, the shortage of donor organs could be overcome if we would be able to transplant organs from other animal species into humans (*xenotransplantation*). Recently, this alternative to allotransplantation has become the subject of widespread medical and commercial interest. Apart from solving the problem of organ shortage, xenotransplantation would offer additional advantages: emergency procedures could be replaced by well-planned routine operations, and organs would be obtained from healthy animals. Unfortunately, so far the history of clinical xenotransplantation has been disappointing [1], and the survival of xenografts in human recipients is too short to be clinically meaningful.

Even when closely related species are used (*concordant* xenotransplantation) as in primate-to-human transplantation, a graft usually does not survive for more than a few days in an untreated host. In addition, a close evolutionary relationship between donor and recipient increases the potential risk of the transmission of pathogens. This risk, together with the limited availability of primates, would favor the use of other donor species. A multitude of animal species have been evaluated for their suitability as donor (ranging from mammals to reptiles and even birds [2–4]), but many may be too different physiologically, or their organs differ in structure from those of humans [5]. Currently, pigs are considered to be the donor animals of choice. Pigs breed well, are commercially available, and have both physiology and sizes of organs and vasculature similar to humans. Also, the use of pigs may generate fewer ethical objections than that of primates.

Unfortunately, the great phylogenetic distance between pig and man induces a violent and rapid rejection reaction, the so-called 'hyperacute (vascular) rejection' (HAR). Species combinations that result in HAR are called '*discordant*'. Hyperacute rejection is antibody- and complement-dependent, and graft failure may occur within a few minutes to hours after transplantation. Unlike the rejection occurring upon concordant transplantation, HAR cannot be prevented by immunosuppressive drugs. Furthermore, even if HAR can be avoided, a few days later, the xenograft may still undergo 'delayed vascular rejection', which is also antibody-mediated. In addition, at later stages (chronic) cellular rejection may occur, characterized by the infiltration of the transplanted organ by human leukocytes. The involvement of white blood cells may depend on binding of their Fc receptors to the Fc portion of antibody bound to the transplanted organ. In a different process,

the recipient's white blood cells will also extravasate into the graft tissue via interaction with selectins expressed on activated endothelium, again leading to cellular rejection. Interestingly, if HAR can be prevented, for example by temporary removal of the anti-donor antibody, a process termed 'accommodation' or 'adaptation' may occur. When the xenoreactive antibodies gradually reappear the graft is still recognized as foreign, but is not rejected. In this case, the transplanted organ may survive for prolonged periods of time. The mechanism for accommodation is not well known. It may be that in this situation the xenoantigens have become masked, or that re-appearance of xenoreactive antibody correlates with a change in repertoire and a decrease in antibody affinity. Alternatively, it is possible that accommodation can occur because the complement reaction has weakened.

In this review, we shall focus on the importance of carbohydrate epitopes ('glycotopes') in the hyperacute rejection that occurs upon transplantation from pig-to-human, and on the merits of strategies to overcome carbohydrate-mediated HAR. The biosynthesis of the major carbohydrate xenoantigen is discussed in detail. The increased knowledge of structure and properties of the key enzyme involved, α1,3-galactosyltransferase, has allowed the design of various novel therapeutic strategies.

2. Natural human anti-carbohydrate antibodies and xenograft rejection

Rejection of a discordant xenograft by an unmodified primate recipient will usually occur within a few hours after transplantation. In most experimental systems, the factors that initiate and sustain a HAR involve binding of a preformed 'natural' antibody to the vascular endothelium of the xenograft. These natural antibodies, found in all higher primates without any known sensitizing event, are absent at birth, but develop within a few weeks or months thereafter. They may arise as a result of colonization of the gastrointestinal tract by microorganisms, and often display a specificity for carbohydrates. In HAR, antibody binding leads to complement activation via the classical pathway. It seems likely that a rapid endothelial cell activation occurs, resulting in hemorrhage and edema of interstitial tissues, and thrombosis of the smaller vessels of the graft [6,7]. Immunopathological analysis showed that the same sequence of events occurs in an ex vivo pig-to-human lung transplantation model [8], in which pig lung was perfused with untreated human blood. Pulmonary dysfunction and failure occurred within 60–150 min of reperfusion. The HAR as observed in this model is similar to that observed in patients receiving organ transplants from an ABO-incompatible donor [9,10].

In the case of allotransplantation involving an ABO-incompatible donor, a natural antibody in the recipient's blood stream binds to the vascular endothelium of the donor organ. This antibody reacts with the A or B histo blood group carbohydrate antigens (see Fig. 1 for structures) present on the surface of the endothelial cells. Certain aspects of the rejection observed in discordant xenotransplantation are similar to this reaction, and therefore it was hypothesized that most likely, natural antibodies are also responsible for HAR of discordant xenografts. The involvement of such antibodies is consistent with the rapid kinetics of HAR, which precludes an evoked immune response. In addition, HAR is characterized by the deposition of antidonor antibodies onto the graft, and it has been observed that the depletion of natural antibodies prior to transplantation prolongs the survival of a vascularized graft. It was proposed that in HAR occurring upon pig-to-human transplantation, a human natural anticarbohydrate antibody might be involved, which recognizes porcine carbohydrate antigens. The latter xenoantigens are different from the ABO determinants, as the reaction is independent of the recipient's blood group status. Cooper and co-workers [11,12] perfused pig heart and kidney with human plasma, and then eluted bound human antibodies from the surface of the organs. In this way, both IgG, IgM and IgA were obtained, which were tested against various synthetic carbohydrates. Surprisingly, it was found that the major proportion of the antibody recognizes a single determinant, a disaccharide of the structure Galα1,3Gal (often termed the 'α-gal' glycotope, Fig. 1) [11–13]. This observation was confirmed by Parker et al. [14].

The anti-pig natural antibody was thus found to have the same specificity as a human anti-Galα1,3-

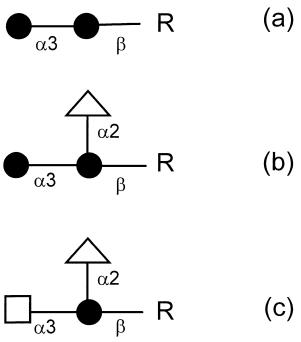

Fig. 1. The Galα1,3Gal glycotope and the blood group B antigen. (a) The major porcine xenoantigen, a disaccharide with the structure Galα1,3Gal. (b) The blood group B antigen, with the structure Galα1,3[Fucα1,2]Gal. (c) The blood group A antigen, GalNAcα1,3[Fucα1,2]Gal. ●, galactose; □, *N*-acetylgalactosamine; Δ, fucose.

Gal antibody that was first described by Galili et al. [15]. This antibody constitutes the most abundant natural antibody in almost all human sera, and in certain individuals it may account for as much as 1% of the total circulating Ig [16]. Initially, it was assumed that this antibody consisted mostly of IgG. However, more recent analyses have established that also IgM and IgA species with the same anticarbohydrate specificity occur in humans [11,12,17,18]. It has been suggested that during HAR of a xenograft it is the binding of IgM which initiates the complement reaction at the surface of the donor organ endothelium, whereas anti-Galα1,3-Gal IgG, by competing with IgM, could play a protective role instead [19]. In addition, Galili [20] showed that the anti-Galα1,3Gal antibody could mediate the antibody-dependent cellular cytotoxicity of human monocytes against porcine cells in tissue culture.

Earlier, it has been shown that the anti-Galα1,3-Gal antibody displays a species-specific distribution [21]. The antibody can be detected in humans, apes and Old World monkeys, but is absent from the circulation of all other mammals, including the New World monkeys. It has been suggested that continuous production of this natural antibody is due to the immune stimulation by gastrointestinal bacteria that express a variety of carbohydrate antigens, including the α-gal glycotope [22]. The presence of the antibody in the human circulation is the main cause of HAR in pig-to-man xenotransplantation.

3. Occurrence of the Galα1,3Gal antigen

The disaccharide Galα1,3Gal is found at the non-reducing terminus of protein- and lipid-bound glycans, and the α-linked galactose is a non-charged alternative to sialic acid as a chain terminator (Fig. 2). The α-gal glycotope is different from the blood group B determinant. Although both structures share the Galα1,3Gal motif, they differ in that a fucosyl residue is an essential part of the B determinant, whereas this fucosyl residue is absent in the α-gal glycotope (Fig. 1). The Galα1,3Gal structure is elaborated by a specific α1,3-galactosyltransferase that is distinct from the blood group B galactosyltransferase (see Section 4).

Galα1,3Gal structures have been detected in a variety of mammalian species. Only cells and tissues derived from catarrhines (humans, apes and Old World monkeys) do not express the α-gal glycotope, and in this respect these species differ from all other mammals [22]. Interestingly, New World monkeys, which separated from the catarrhines only ∼40 million years ago, continue to express the epitope. These data are consistent with earlier results obtained by Spiro and Bhoyroo [23], who showed that the glycoprotein thyroglobulin displays a species-specific α1,3-galactosylation of its *N*-glycans. It seems, therefore, that the expression of α-gal glycotopes is complementary to the distribution of the natural anti-Galα1,3Gal antibody [21].

Most non-mammalian species that have been examined to date appear not to express Galα1,3Gal glycotopes, with the possible exception of the cobra *Naja naja kaouthia* [4]. Carbohydrate analysis of cobra venom glycoproteins showed that *O*-linked polylactosaminoglycans of mucin-like proteins are terminated by α1,3-linked galactose or α1,3-galactosylated Lewis^x structures [24,25]. The egg glycoproteins (hyosophorins) of several teleost fish species were also found to contain glycans terminated with the structure Galα1,3Gal [26].

The α-gal glycotope is found both on mammalian tissues and cells in tissue culture. In the mouse, *Griffonia simplicifolia* I-B4 (GSI-B4) lectin staining has detected Galα1,3Gal structures in a variety of organs and cell types, with the exception of liver [27]. Expression levels of the Galα1,3Gal glycotope in mouse liver seem to be strain-dependent, though, as DBA/2

mice unlike Balb/c show a weak positive reaction [28]. In contrast, using GSI-B4 lectin, both Cooper et al., and Sandrin and McKenzie, found that the parenchyma of porcine liver contains α-linked galactose residues [28,29]. However, more recently Oriol et al. showed that, although pig hepatocytes react with GSI-B4, the reaction cannot be abolished by α-galactosidase treatment of the cells. Most likely, the observed lectin binding is an artifact due to the presence of the asialoglycoprotein receptor at the cell surface [4]. In another lectin- and immunostaining analysis of porcine tissues, Oriol et al. [30] detected various carbohydrate antigens on endothelial cells. Vascular endothelium showed a strong reactivity with reagents specific for α-galactosylated structures, although in addition, sialylated structures are present. The α-gal glycotope was also present on erythrocytes, leukocytes and platelets. In general, finer details of the Galα1,3Gal expression pattern may differ from species to species (e.g. between pig and mouse), but consistently, high-level expression is found on the endothelial cells of all capillaries, arterioles and arteries, the first point of contact between antibodies and donor organ.

Cairns et al. [31] and Vaughan et al. [32] have detected on pig vascular endothelium both Galα1,3-Gal containing glycoproteins and glycosphingolipids. Detailed structural analyses of the xeno-epitope have been carried out by Samuelsson and colleagues [33–35], who showed that the major α1,3-galactosylated glycosphingolipid on porcine endothelium is ceramide-pentahexoside (Galα1,3Galβ1,4GlcNAcβ1,3-Galβ1,4Glc-ceramide, see Fig. 2). The protein-linked glycans contain the Galα1,3Galβ1,4GlcNAc trisaccharide at their non-reducing terminus (Fig. 2). Interestingly, the major endothelial cell surface glycoproteins carrying the Galα1,3Gal glycotope were identified as Von Willebrand factor, and various integrins [36]. More sensitive procedures, however, detected multiple additional α1,3-galactosylated cell surface proteins, expressed at lower levels [28,37].

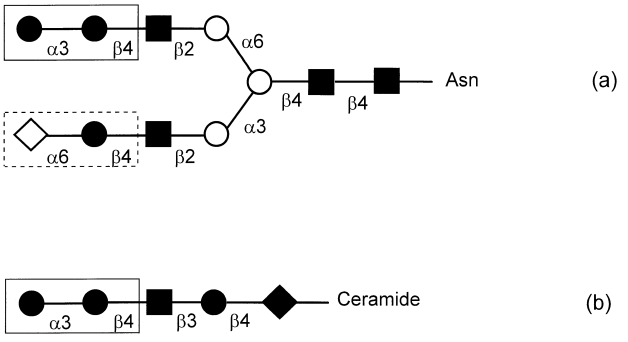

Fig. 2. Terminal structures of glycoprotein and glycolipid glycans. (a) A diantennary *N*-glycan. In mammals, complex-type and polylactosamine chains are often terminated by sialic acid or α1,3-linked galactose. (b) Ceramide-pentahexoside, Galα1,3-Galβ1,4GlcNAcβ1,3Galβ1,4Glc-ceramide. Terminal structures shown are the α-gal glycotope (Galα1,3Gal) (boxed) and the NeuAcα2,6Gal structure (boxed with dashed line). Alternatively, *N*- and *O*-glycans can be terminated by blood group determinants (Galα1,3[Fucα1,2]Galβ1-R, GalNAcα1,3[Fucα1,2]-Galβ1-R, or Fucα1,2Galβ1-R), polysialic acid, or sulfate groups. The same terminal structures can be found on both glycoprotein *N*- and *O*-glycans and glycolipids. ■, *N*-acetylglucosamine; ○, mannose; ●, galactose; ◆, glucose; ◇, sialic acid; △, fucose.

4. Synthesis of the Galα1,3Gal xenoantigen by α1,3-galactosyltransferase

4.1. The enzyme α1,3-galactosyltransferase

Xenoantigens that contain the Galα1,3Gal structure are produced by UDP-Gal:Galβ1,4GlcNAc α1,3-galactosyltransferase (α3GalT, EC 2.4.1.87). This enzyme catalyzes the reaction:

$$\text{UDP-Gal} + \text{Gal}\beta 1,4\text{GlcNAc-R} \xrightarrow{Mn^{2+}} \text{Gal}\alpha 1,3\text{Gal}\beta 1,4\text{GlcNAc-R} + \text{UDP},$$

in which R may be glycoprotein or glycolipid [38].

N-Acetyllactosamine (Galβ1,4GlcNAc) is the preferred substrate for the enzyme; lactose (Galβ1,4Glc) is also recognized as an acceptor, but its K_m is 6–7-fold higher. Both protein- and lipid-linked lactosaminoglycans are excellent substrates for α3GalT. Importantly, the enzyme cannot transfer to fucosylated substrates such as the blood group H structure (Fucα1,2Galβ1,3/4GlcNAc), and therefore is unable to produce the blood group B determinant. In this respect, it is different from the blood group B α1,3-galactosyltransferase. Also, the Lewis^x structure (Galβ1,4[Fucα1,3]GlcNAc) is not an acceptor substrate for α3GalT. Fig. 3 shows the reaction products of the blood group B transferase and α3GalT.

A good glycolipid acceptor substrate for α3GalT is

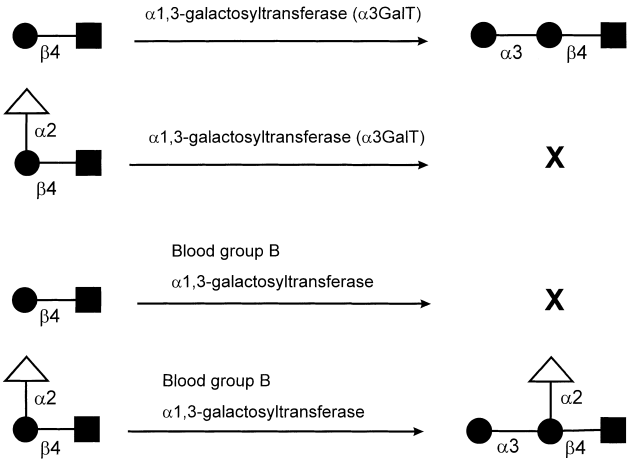

Fig. 3. Reactions catalyzed by α3GalT and the blood group B α1,3-galactosyltransferase. Fucosylated acceptors such as Fucα1,2Gal or Galβ1,4[Fucα1,3]GlcNAc cannot be α1,3-galactosylated by α3GalT. In contrast, a non-fucosylated acceptor, such as Galβ1,4GlcNAc, is not a substrate for the blood group B transferase. The X indicates a reaction that does not occur. For symbols, see Fig. 2.

lacto-*N*-*neo*tetraosylceramide (Galβ1,4GlcNAcβ1,3-Galβ1,4Glc-ceramide), but the enzyme cannot use lactosylceramide (Galβ1,4Glc-ceramide). It is not yet known whether the protein portion of a glycoprotein acceptor can interact with α3GalT, and in this way affect galactosyl transfer to the glycans it carries. Such acceptor protein preference might explain the observation that only a limited number of major, α1,3-galactosylated glycoproteins is detected on porcine endothelial cells (see above).

4.2. The α3GalT gene family

Genes and cDNAs encoding α3GalT have been cloned from several different species [39–44]. The bovine α3GalT cDNA was the first to be isolated [39], and the properties of the corresponding enzyme have formed the basis for subsequent studies. The enzyme was purified from thymus by use of affinity chromatography on UDP-Sepharose [38], and the α3GalT cDNA was then isolated from a bovine cDNA library by immunoscreening using an anti-α3GalT antibody and α3GalT-specific oligonucleotide probes [39]. A 1836-bp cDNA was cloned that encoded a 368-amino acid protein. The coding sequence predicted a type II transmembrane protein, consisting of a 6-amino acid amino-terminal cytoplasmic tail,

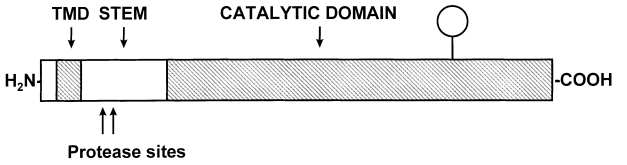

Fig. 4. Schematic of the protein domain structure of α3GalT. The cytoplasmic tail, the transmembrane domain (TMD), the stem region and the catalytic domain are indicated. A single *N*-glycan is attached to the catalytic domain at Asn-293 (indicated as ○, not drawn to scale). Proteolysis in the stem region of the enzyme (arrows) would result in the secretion of a truncated, soluble, enzymatically active protein.

a transmembrane domain (signal anchor sequence) of 19 residues, and a large, carboxy-terminal catalytic domain of approximately 280 amino acids (see Fig. 4). The catalytic domain is linked to the transmembrane domain via a ~60-amino acid hydrophilic linker apparently devoid of secondary structure, termed the 'stem region'. More recent studies [42] have confirmed that the α3GalT catalytic domain is likely to be contained in the carboxy-terminal ~286 amino acid residues.

Other mammalian α1,3-galactosyltransferase orthologs: cDNAs encoding mouse α3GalT were obtained from F9 cells by expression cloning [40], and by homology screening of a cDNA library using the bovine cDNA as a probe [41]. The two murine cDNAs predict polypeptides that differ in the length of the stem, as a result of alternative splicing. Comparison of the full-length murine protein, derived from the genomic sequence, with bovine α3GalT revealed a 73% identity at the amino acid level. A large number of differences are present in the stem region, whereas a high degree of similarity (79% identical amino acids in 286 residues) is found in the carboxy-terminal portion of the enzyme which contains the putative catalytic domain.

Homologous sequences from marmoset (a New World monkey) [42] and pig [43,44] were cloned by library screening using the murine α3GalT cDNA as a probe. Again, there is a high degree of similarity in the carboxy-terminal 250 amino acid residues when this region is compared with α3GalTs from other mammalian species. Partial α3GalT sequences were obtained from various species of primates by use of PCR [45]. The human genome was found to contain two homologs of the α3GalT gene [46,47]. However,

both genes are pseudogenes, and apparently neither gives rise to a transcript (see below).

Extensive sequence similarities exist between the mammalian α1,3-galactosyltransferase orthologs [39–45], the ABO blood group transferases [48,49], Forssman α1,3-GalNAc-transferase [50], and related pseudogenes [46,51]. Together these genes form the α3GalT gene family (cf. Breton et al. [52]). The extent of amino acid sequence conservation between the mammalian α3GalT orthologs is in the order of 73–88%. The similarity with the human blood group transferases is of special interest in view of the shared chromosomal localization of these genes with one of the human α3GalT homologs. In addition, their genomic organization is similar: both the human ABO genes and the (murine) α3GalT gene have their protein coding sequence distributed over multiple exons, while the major portion of the coding sequence is present on a single large exon [41,53]. The latter exon is likely to encode almost all (∼230 amino acids) of the catalytic domain (Fig. 4). These observations point to a close evolutionary relationship between α3GalT and the blood group transferases.

4.3. Species-specific expression of α3GalT

The expression of α3GalT is usually inferred from direct enzyme activity assays, or from the detection of α3GalT transcripts. The expression of α3GalT seems to be restricted mainly to mammals, although it is possible that related enzymes are present in certain species of fish and snakes. Earlier, we have shown that although the human genome contains gene sequences homologous to mammalian α3GalT [39], no transcripts are detected in human cell lines in tissue culture. Similarly, no α3GalT transcripts are detected in a number of cell lines from apes and Old World monkeys. Most likely, mutation(s) have occurred in the upstream regulatory sequence of the α3GalT gene in catarrhines, resulting in a suppression of transcription.

In contrast, enzyme expression can be detected in most mouse and rat tissues and organs, as determined by direct enzyme activity assays, and RT-PCR. However, mouse liver seems to be devoid of α3GalT both on enzyme assay and transcript detection (Joziasse, D.H., unpublished observation, and [54]). In addition, murine male germ cells do not contain α3GalT transcripts [41,55]. In mice in which the α3GalT gene has been inactivated via homologous recombination, no enzyme activity is detectable, and no Galα1,3Gal structures can be detected on the various tissues [56,57]. This is consistent with our earlier observation that murine α3GalT is encoded on a single gene [58]. In general, it appears that the expression of α3GalT activity parallels the species-specific expression of α-gal glycotopes.

An α1,3-linked galactose is also present in trihexosylceramide, which has the structure Galα1,3-Galβ1,4Glc-ceramide. However, as α3GalT cannot use lactosylceramide (Galβ1,4Glc-ceramide) as a substrate (see 4.1), trihexosylceramide must be the product of a different α1,3-galactosyltransferase, as detected in rat spleen and bone marrow [59]. Also, a globoside containing the structure Galα1,3Galα1,4-R, and more extended globosides with internal repeats of α1,3-linked Gal residues, are present in rat small intestine and in rat pheochromocytoma cells (PC12h) [60]. The α1,3-galactosyltransferase involved in the synthesis of such glycosphingolipids has been characterized in detail [61]. It may be related to α3GalT, but as yet no sequence information is available.

The lack of α3GalT expression in humans, apes and Old World monkeys does not seem to have a deleterious effect. On the other hand, transgenic mice in which the functional α3GalT gene has been inactivated by homologous recombination remain healthy and appear normal, but do develop eye cataracts [57]. It is possible that the occurrence of this problem is limited to the mouse. If so, one may wonder why most mammalian species have maintained the functional gene. Various glycosylation polymorphisms are known (e.g. in the human ABO blood groups, and the expression of certain α1,2-fucosyltransferases), but do not seem to be connected with an evolutionary advantage. Curiously so far no polymorphism has been detected in the expression of α3GalT in pigs [4,10,62], but it is possible that there are animals in which one of the two copies of the α3GalT gene is inactive. Such heterozygosity would go unnoticed, as expression from a single, active gene copy is anticipated to generate sufficient α3GalT activity to confer the α-gal$^+$ phenotype on pig glycoproteins. Therefore, there is no reason to expect se-

lection against heterozygotes per se. Theoretically, breeding of these heterozygotes, once identified, might generate homozygous null mutants. However, it remains to be seen if these null mutants would be viable, and develop normally. Failure of these animals to reach adulthood would, under natural conditions, affect the breeding efficiency of heterozygotes, and ultimately select for $\alpha 3GalT^{+}$ homozygosity.

Conversely, it has been suggested that host–pathogen interactions have provided the selective pressure to inactivate the α3GalT gene in the catarrhines. Two mechanisms can be considered. Non-reducing terminal saccharide structures are functionally important in the binding of pathogenic microorganisms to mammalian cells. A change in the glycan structures that are expressed at the mammalian cell surface may abolish specific pathogen binding, and thus protect the host. Also, an individual with altered glycosylation might be able to produce a different spectrum of natural anticarbohydrate antibodies, which may provide protection against specific pathogenic bacteria and viruses (cf. Section 7, and [63, 64]). An example of such situation may be found in the ABO polymorphism [65]. Neither of the two mechanisms, however, seems sufficient to explain how heterozygosity at the α3GalT locus might have conferred an evolutionary advantage, as the inactivation of both gene copies is required for a change in cellular glycosylation. It is conceivable, though, that α-gal^{-} offspring may show a higher breeding efficiency, which would select for $\alpha 3GalT^{-}$ homozygosity.

5. Molecular evolution of the α3GalT gene family: inactivation of the α3GalT gene

In the human genome, α3GalT is represented by two pseudogenes, HGT-2 and HGT-10 [46]. One of these genes, HGT-2, has the characteristics of a processed pseudogene, the other (partial) gene, HGT-10, is likely to be the missing 5′ portion of a human α3GalT sequence that was isolated by Larsen et al. [47]. The latter gene, like HGT-2, contains multiple insertions, deletions, and point mutations that result in premature stop of translation. It contains several introns, and is likely to be the remnant of the once functional gene in humans. In view of the shared localization of human α3GalT homolog HGT-10 and the ABO locus on chromosome 9q34 [66], the observed sequence similarity between the ABO blood group transferases and α3GalT, and their similar genomic organization, it is hypothesized that these genes originate from a single ancestral gene (Fig. 5). After tandem duplication of the ancestral gene ~400 million years ago [65], one gene copy might have evolved into the ABO genes, and the other one into the human α3GalT gene sequence on chromosome 9 represented by HGT-10. At some point in time, after the divergence of New World monkeys from the catarrhines, the human α3GalT gene may have given rise to an mRNA that, after reverse transcription, was inserted into chromosome 12.

It has been shown by gene cloning and PCR analysis that during the course of evolution, the α3GalT gene has been inactivated in the catarrhines (Old World monkeys, apes and man), although it continues to be functionally expressed in most other species of mammals including the New World monkeys [22,45–47]. Homologs of the α3GalT gene are present in the genome of apes and Old World monkeys, but they are pseudogenes like their human counterparts [46]. Their coding sequence contains multiple frame shift mutations; therefore, these genes are not anticipated to encode an enzymatically active

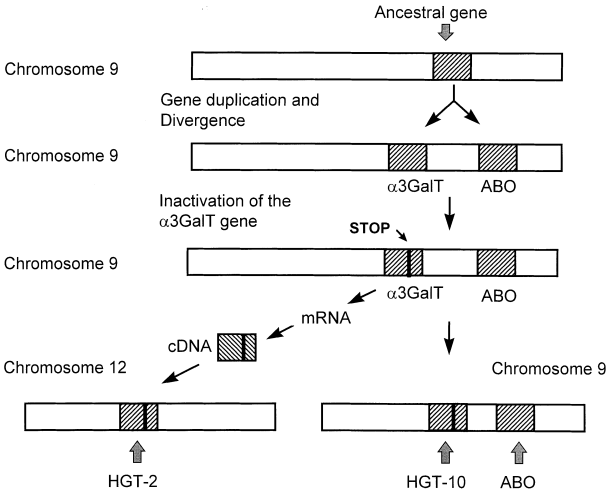

Fig. 5. A model of the evolution of the α3GalT gene family and the inactivation of the α3GalT gene in catarrhines. Duplication of the ancestral gene may have occurred ~400 million years ago. Subsequent divergence of the two gene copies has given rise to the ABO blood group genes and α3GalT.

protein. Focusing on these frameshift mutations, Galili concluded that gene inactivation in apes was a relatively recent event, and that separate gene inactivating events have occurred in the Old World monkeys on the one hand, and in apes and humans on the other, after the two groups diverged from each other [45].

However, we prefer a simpler, previously suggested model [67] in which the α3GalT gene was inactivated in the lineage of the catarrhines by a single event (compare Fig. 5). This event, the introduction of a stop codon at amino acid position Tyr-263 (bovine sequence) occurred after the divergence between catarrhines and the New World monkeys, but prior to further divergence of the catarrhines. DNA sequence analysis has shown that the mutation to TAG interrupts the coding sequence in all Old World monkey and ape sequences analyzed thus far [45,67]. The same mutation is present in the partial human α3GalT pseudogene sequence isolated by Larsen et al. [47]. This sequence is likely to be part of human pseudogene HGT-10, located on chromosome 9 [46], presumably the original, once functional α3GalT gene in hominids. The nonsense mutation leads to a premature termination of translation, resulting in gene inactivation. The mutation is absent in all species that express an active α3GalT homolog (New World monkeys, cow, pig and mouse), where the codon reads TAC or TAT (Tyr). Importantly, the same mutation is present in the human processed pseudogene HGT-2, which may suggest that transcription of the gene continued for a certain period of time even though translation of the mRNA would have yielded a truncated, enzymatically inactive protein. Such transcription of a glycosyltransferase pseudogene is similar to what is observed today for the blood group type O. We have estimated the age of the processed pseudogene HGT-2 to be ∼25 million years [46]. As the catarrhines and New World monkeys diverged ∼40 million years ago, this would mean that α3GalT inactivation has occurred between 25 and 40 million years ago. At the time when HGT-2 originated, the source gene continued to be transcribed, but must already have contained the stop codon at amino acid position 263. An additional mutation in the regulatory sequences of the source gene must have occurred somewhat later in evolution, resulting in the current lack of transcription in the catarrhines.

From the sequence differences between the human pseudogene HGT-10 and the New World monkey sequences (4.6–7.0%), it can be calculated that gene inactivation may have taken place between 22 and 32 million years ago [45]. Most of this period overlaps with the 15-million year window as defined above. This would be consistent with a scenario in which gene inactivation through the insertion of a stop codon occurred prior to the divergence of apes from Old World monkeys (20–28 million years ago), and explain the presence of this stop codon in all α3GalT pseudogenes.

6. Strategies to prevent HAR in discordant xenotransplantation

Various approaches have been considered to prevent hyperacute rejection upon organ transplantation from pig to humans. We can distinguish a suppression of the immune reaction, the induction of immune tolerance in the recipient, or a modification of the tissues of the donor. The HAR reaction may be prevented by blocking, or depletion of the natural anti-Galα1,3Gal antibody. With respect to a modification of the donor tissue, an alteration of the glycosylation phenotype of the tissue is being considered in order to suppress the expression of the Galα1,3-Gal glycotope. Another approach is based on the expression of human complement-regulatory proteins on donor endothelial cells.

6.1. Antibody depletion

In case of allotransplantation across the ABO blood group barrier, depletion of the anti-A (or anti-B) antibody is required prior to transplantation. It has been shown clinically that removal of the anti-blood group natural antibody does enable ABO incompatible transplantation [29]. It is possible to remove all Ig's by plasmapheresis, but this may leave the recipient susceptible to infection and thrombogenic disorders. This is why a specific removal of the anti-donor antibodies is preferred. Antibody can be depleted by extracorporeal immunoadsorp-

tion on affinity columns that carry the blood group antigen of interest. The temporary reduction in antibody levels prevents HAR, and may result in accommodation.

A similar approach has been proposed in the case of pig-to-human xenotransplantation. Here, immunoadsorption can be accomplished on affinity columns carrying the major pig xenoantigen, Galα1,3-Gal-R. It is interesting to note that the Galα1,3Galβ1,4Glc trisaccharide glycotope (type 6) seems to have a higher affinity than the Galα1,3-Galβ1,4GlcNAc (type 2) structure [68], even though it is well known that α3GalT prefers the type 2 structure *N*-acetyllactosamine as an acceptor over lactose (see above). It is not clear, though, to what extent the presence of the hydrophobic spacer that links the oligosaccharide to the column may affect the binding properties of the oligosaccharides. Furthermore, it has been shown that a recombinant mucin such as PSGL-1, carrying multiple copies of the Galα1,3Gal glycotope, is also an efficient immunoadsorbant [69]. The glycans attached to this glycoprotein may structurally be more similar to the natural xenoantigen than the oligosaccharides linked to affinity matrices, and may offer a more varied range of structures. As the anti-α-gal antibody is heterogenous in its preference for di-, tri-, or pentasaccharide structures [70], the mucin may thus allow a more complete antibody adsorption. Alternatively, xenoreactive antibodies can be removed via a perfusion of the recipient's blood through pig organs [71]. The efficiency of immunoadsorption in reducing serum cytotoxicity against porcine endothelial cells in tissue culture was shown in various in vitro studies [72–74]. Lastly, the use of anti-idiotype antibodies has also been proposed as a means to reduce the binding of xenoreactive antibodies [75]. Apparently, the xeno-reactive natural antibodies have a limited idiotope diversity, which is why combination of only a few anti-idiotype antibodies is sufficient for blocking.

Of course, it remains to be investigated if temporary depletion of the antibody will be sufficient to ensure long-term transplant survival. Upon termination of the treatment, there is a rapid reversal (within 4 days) of natural antibody titers. However, it is hoped that if hyperacute rejection can be prevented, accommodation may occur as in ABO incompatible transplantation. A continued pharmacologic immunosuppressive therapy will be necessary, though, to prevent the development of cellular rejection, and perhaps to reduce the production of new antibody, while accommodation is taking place.

6.2. 'Neutralizing' oligosaccharides

The alternative to immunoadsorption is the intravenous infusion into the recipient of oligosaccharides that mimic the xenoantigen, a treatment which has to be applied both prior to and following xenotransplantation in order to be effective. This method, in which a blood group trisaccharide was infused into the recipient, has been successful in allowing ABO-incompatible cardiac allografting in the baboon [76,77]. In a similar way, intravenous infusion of Galα1,3Gal oligosaccharides was shown to reduce baboon serum cytotoxicity to transplanted pig hearts [74,78], and to delay HAR [79]. The same protective effect was observed when pig kidney (PK-15) and vascular endothelial cells in tissue culture were exposed to human or baboon serum to which Galα1,3-Gal oligosaccharides had been added [74,80]. Cytotoxicity was not abolished by a variety of other carbohydrates, which underlines that the exact structure of the Galα1,3Gal oligosaccharides used for blocking or immunoadsorption should closely resemble the porcine xenoantigen. It is likely that porcine tissues contain the disaccharide at the non-reducing terminus of both glycoprotein and glycolipid glycans (see above), as part of the structure Galα1,3-Galβ1,4Glc(NAc)β1-R. It was found that both the

Table 1
'Neutralizing' oligosaccharides

Inhibitor oligosaccharide	Concentration of oligosaccharide (μM)	
	Human serum	Baboon serum
Galβ1-R	> 10 000	> 10 000
Galα1,2Galβ1-R′	7 000	10 000
Galα1,3Gal	386	301
Galα1,3Galβ1,4Gal	163	141
Galα1,3Galβ1,4GlcNAc	27	31

The concentration of oligosaccharide corresponds to the concentration that is required for a 50% inhibition of cytotoxicity of unmodified human or baboon serum against pig PK-15 cells [81]. R represents a 1,3- or 1,4-linkage to an underlying sugar residue. R′ is the hydrophobic linker $-O(CH_2)_3$-$NHCOCF_3$.

length of the inhibitor, and the proper structure of both the reducing and the non-reducing end of the oligosaccharide are important for achieving sufficient inhibitory activity [72,81]. Essential is the correct linkage type (i.e. α1,3) between terminal and subterminal sugar residue (see Table 1). Compounds containing Galα1,2Gal or Galα1,6Gal structures are less efficient, and modification of the linkage type of the non-reducing terminal sugar from α to β completely abolishes the inhibition of cytotoxicity. Also, the trisaccharides Galα1,3Galβ1,4GlcNAc and Galα1,3Galβ1,4Glc are at least 10-fold more efficient inhibitors than the disaccharide Galα1,3Gal. These observations point to a rather narrow specificity of the natural human antibody.

The various compounds used in clinical studies are needed in rather high amounts (grams to kilograms). They can be produced by organic or chemo-enzymatic synthesis, and glycosyltransferase-catalyzed synthesis in particular is highly effective. This method takes advantage of the availability of recombinant α1,3-galactosyltransferase (produced in insect cells [82], or bacteria [83]) and a variety of other recombinant glycosyltransferases [84], and of the absolute regio- and stereo-selectivity of these enzymes. For large-scale synthesis, it is essential to be able to regenerate expensive nucleotide sugars in situ [85,86], preferentially in a one-pot synthetic reaction. Oligosaccharide inhibitors may also be produced in alternative systems. Recently, it has been proposed to use recombinant bacteria, expressing the appropriate glycosyltransferases, as micro-fermenters to produce the desired oligosaccharides (R. Geremia, personal communication). Various glycosylhydrolases have also been applied in oligosaccharide synthesis. These enzymes show a good stereo-selectivity, and do not require nucleotide sugars, but are of limited regioselectivity [84,86].

Alternative approaches have been developed in which high-molecular weight natural glycoproteins are used as blockers (e.g. pig stomach mucin), although there is a risk that these compounds may induce an immune reaction, and perhaps may lead to the formation of harmful antigen–antibody complexes. Another disadvantage is that the active moieties are not always well defined. In an improved procedure the *O*-glycans of these mucins have been prepared by β-elimination, and were shown to be efficient inhibitors of primate serum cytotoxicity against pig PK-15 cells in vitro [87].

Importantly, there is a large difference in affinity between IgG and IgM, both components of the natural xenoreactive antibody. It has been found that whereas monovalent compounds in micromolar concentration can block IgG, more efficient blockers are needed to inhibit IgM binding. For this purpose, specific multivalent compounds have been developed by Bovin and co-workers [88], made of spacered oligosaccharides attached in a multivalent fashion onto a polyacrylamide backbone (activated polymer, poly-(4-nitrophenylacrylate)). These compounds were found to be highly efficient blockers of IgM binding, as compared to monovalent compounds. Also, adsorption columns carrying the multivalent polyacrylamide derivatives were constructed, and were shown to be efficient immunoadsorbants [72].

A quite different approach towards the induction of immune tolerance was reported recently [89]. By recombinant retroviral transduction of the bone marrow, an $\alpha 3GalT^{+}$ genotype was induced in $\alpha 3GalT^{-}$ knock-out mice, which as a result of the gene inactivation produce a natural anti-α-gal antibody, like primates. The expression of the α-gal glycotope in their bone marrow effectively abolished the production of the natural antibody [89]. It is conceivable that a similar treatment of a human recipient prior to transplantation of a pig organ might induce immune tolerance towards the carbohydrate xenoantigen on porcine tissues.

6.3. Modification of the donor organ

Even though in many respects HAR in ABO incompatible transplantation is similar to that observed in pig-to-human organ transplantation, it may be less severe as the complement reaction is modulated by complement inhibitory proteins. However, in case of xenotransplantation, the modulation is less efficient as complement-regulatory proteins tend to be species-specific. This has led to the idea of producing transgenic pigs that express proteins such as human DAF, an inhibitor of the complement reaction, on their endothelial cells [90,91]. Indeed, this strategy has prevented the occurrence of HAR, both in vivo (cardiac transplants to baboons [92]), and in vitro (protection of hDAF-expressing porcine

endothelial cells against the combined action of human xenoreactive antibodies and complement [90,91]).

To modify the glycosylation of the donor tissue, a simple approach would be to treat the tissue with a glycosidase, such as α-galactosidase. In case of a vascularized organ one could thus remove most of the Galα1,3Gal from the endothelium prior to transplantation. The disadvantage, however, is that most likely the α-gal glycotopes will rapidly re-appear as a result of de novo synthesis, and transport to the cell surface. It is conceivable that expression of α-galactosidase within the Golgi apparatus of a transgenic animal would provide a more lasting effect.

Alternatively, it has been considered to modify glycosyltransferase expression via gene inactivation in transgenic animals, or by altering the expression of the relevant gene by modification of the promoter. Here, a major target would be the α3GalT gene. Suppression of its expression would result in a global modification of the cell surface, and induce an α-gal$^-$ phenotype. Indeed, an analysis of transgenic mice that were homozygous null-mutants for α3GalT showed the absence of the α-gal glycotope in all tissues examined [56,57]. It is anticipated that, similarly, in pig null mutants all α1,3-galactosylation will be abolished. However, it may be preferable to limit α3GalT suppression to certain tissues only. Cell-type specific gene ablation could be accomplished using *Cre*-recombinase, or by specific mutations in the α3GalT promoter. It has been reported recently that α3GalT gene expression in porcine endothelial cells is regulated in a cell-type specific way [93]. Activation of endothelial cells resulted in a transient decrease in gene transcription, and correlated with decreased α3GalT protein stability.

Another possibility to suppress the α-gal glycotope would be the production of transgenic pigs that express a glycosyltransferase competing with the action of α3GalT, or masking the α-gal glycotope. It is known that terminal Galβ1,4GlcNAc sequences serve as an acceptor for multiple glycosyltransferases including α3GalT. Certain glycosyltransferases directly compete with α3GalT for available acceptor sites (i.e. an alternative sugar takes the place of the α1,3-linked galactose residue). For example, if α2,3-sialyltransferase is expressed in an early Golgi compartment, it will attach sialic acid residues to the 3-position of terminal galactose residues on complex-type *N*-glycans, thus blocking α1,3-galactosylation. Indeed, overexpression of α2,3-sialyltransferase in transfected porcine endothelial cells in tissue culture reduced both their binding of GSI-B4 lectin and the sensitivity to lysis mediated by complement and human serum [94]. These observations differ from preliminary data reported earlier by Sharma et al. [95], but as experimental details are not available it is not clear if these studies can be compared.

Indirect competition is often the result of the stringent acceptor substrate specificity of the glycosyltransferases. Substitution of the α3GalT acceptor (Galβ1,4GlcNAc-R) with another sugar residue may hinder the action of α3GalT; examples are the competition of α3GalT with α2,6-sialyltransferase, with α1,2-fucosyltransferase H, or with an α1,3-fucosyltransferase. Competition between α3GalT and the H transferase in transfected CHO cells led to an increased formation of the H antigen, at the expense of the α-gal glycotope. Similarly, the expression of the human H fucosyltransferase in transgenic mice and pigs resulted in a high expression of the H antigen, together with a strong reduction in the expression of the Galα1,3Gal structure [95]. This resulted in a decreased binding of xenoreactive antibodies to endothelial cells of the transgenic animals, and in a protection from complement-mediated lysis. Similar observations had earlier been made on xenogeneic cells in tissue culture that expressed human α1,2-fucosyltransferase [96].

7. Protective roles of the natural anti-Galα1,3Gal antibody

The anti-Galα1,3Gal antibody is of clinical relevance, as it is the major antibody directly responsible for HAR, and is also involved in antibody-dependent cellular rejection. A lasting depletion of this antibody from the human circulation therefore seemed to be a promising strategy to prevent HAR and thus to facilitate xenograft transplantation. Unfortunately, it is likely that the anti-Galα1,3Gal antibody has a role in providing protection against various pathogens that carry Galα1,3Gal structures on their surface. These pathogens may include parasites, such as Trypano-

somes and *Leishmania*, bacteria, and various enveloped viruses.

In *Leishmania* and Trypanosomes, indirect evidence suggests that Galα1,3Gal structures may be present both on glycolipids and glycoproteins [97,98]. Also, in *Leishmania*, lipophosphoglycans may contain internal α1,3-linked Gal in the glycan core which is linked to a phosphatidyl-inositol anchor [99]. These structures are a potential target for the natural anti-α-gal antibody.

Retroviruses produced in non-primate species frequently carry glycoproteins on their envelope with carbohydrate moieties that reflect the biosynthetic capacities of the host organism. For example, retroviral glycoproteins produced in mouse and porcine cells are likely to be α1,3-galactosylated. Indeed, the anti-α-gal antibody was shown to be responsible for complement activation and virolysis of murine derived retroviruses in primate sera [64]. Similarly, virus particles produced in cells transfected with α3GalT will carry α-gal glycotopes on their surface, and as a result become sensitive to complement-mediated lysis in contact with human serum [100]. Once introduced into the human blood stream, these virus particles will be opsonized by the anti-Galα1,3-Gal antibody, and be inactivated. A similar mechanism would protect humans against pig endogenous retroviruses that can be mobilized from the pig genome, and be secreted from a transplanted pig organ into the recipient. However, the protection thus afforded against the pathogen may fail if anti-α-gal antibody is permanently removed from the blood stream. In particular, the elimination of human B cell clones producing the anti-Galα1,3Gal antibody may constitute a serious risk, by leaving the recipient unprotected.

Another risk is the production of virus particles that lack Galα1,3Gal structures. It has been shown that a subset of human cell lines in tissue culture (thus, in the absence of the anti-Galα1,3Gal antibody) can be infected by pig endogenous retroviruses [63]. Moreover, co-cultivation of human cells with porcine endothelial cells resulted in a broader subset of tissue culture cell lines that could be infected. Following passage through human cells, the pig retroviruses could rescue a Moloney retroviral vector, and acquired resistance to lysis by human complement. So, if pathogens are produced from the tissues of an animal which does not express Galα1,3Gal glycotopes, they may go unnoticed by the immune system. Thus, suppression of α1,3-galactosylation in porcine organs may increase the possibility of interspecies viral transmission. In this way, complement-mediated inactivation of retroviruses in human serum triggered by anti-Galα1,3Gal, a unique type of natural immunity, would be rendered non-functional [64,101]. This factor will need to be taken into account by the time the technology becomes available to produce transgenic pigs lacking α3GalT through recombination in embryonic stem cells, or by nuclear transfer methodology. The transgenic mice in which α3GalT has been inactivated, and which as a result develop a natural anti-α-gal antibody, will be an important model system to assess the importance of anti-α-gal in immunity against retroviruses [56].

8. Future directions

The identification of the major carbohydrate antigen mediating HAR in pig-to-human rejection, together with the cloning and the characterization of the α1,3-galactosyltransferase responsible for its production, has opened new avenues for the prevention of HAR. The production of recombinant α3GalT allows the synthesis of ligands for immunoadsorption, and for specific intravenous carbohydrate therapy. An analysis of α3GalT gene expression patterns has helped to explain the distribution of the anti-Galα1,3Gal antibody, and to understand the natural, complement-mediated immunity against interspecies retroviral transmission. The cloning of α3GalT genomic sequences has already allowed to generate animals devoid of enzyme expression by homologous recombination.

Although carbohydrate epitopes other than Galα1,3Gal might play a role in rejection, with the availability of carbohydrate-directed therapy it is likely that the HAR will soon be overcome. It remains a matter of debate, though, which approach is most suitable here. Both a lasting suppression of the production of anti-Galα1,3Gal antibodies in human recipients and the long-term effects of pharmacologic immunosuppression are undesirable. We believe that, preferentially, efforts should be directed both at the donor and the recipient. Most promising

seems a combination of strategies such as the expression of human complement inhibitors, together with a local modification of the glycosylation of the donor tissue, and a temporary removal or blocking of the anti-Galα1,3Gal antibody. Once delayed and chronic effects, which most likely involve interactions different from anti-α-gal binding, are under control, one could hope to reach the stage of accommodation which might guarantee a long-term survival of the transplanted organ. The former processes will be the next problems to be tackled in the field of pig-to-human xenotransplantation.

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