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MEDICAL REVIEW

Recent Strategies to Overcome the Hyperacute Rejection in Pig to Human Xenotransplantation

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Due to the ever increasing shortage of suitable human donors, alternative strategies are sought to moderate the current discrepancy between the number of executable and required transplantations. Xenotransplantation (i.e., the transplantation of organs [tissues or cells] between different species) appears to be a reasonable solution. However, various problems (immunological, physiological, infectious-microbiological, ethical-juridicial) seem to be associated with xenotransplantation. One of the most formidable barriers to xenotransplantation is the phenomenon of hyperacute rejection that may lead to the destruction of the transplanted vascularized organ in a few minutes to hours. In the pathogenesis of hyperacute rejection, xenoreactive antibodies and the complement system appear to be of primary importance. Various methods can be applied to prevent hyperacute rejection; both the recipient and the donor can be treated. In this brief review, the author attempts to present a synopsis of the possible therapeutical interventions to prevent hyperacute rejection..

INTRODUCTION

Based on the ever increasing shortage of suitable human donor organs, alternative solutions are sought to diminish the difference between the number of required and executable organ transplantations. Xenotransplantation, i.e., the transplantation of organs (tissues or cells) between different species seems to be a possible solution, since it could provide unlimited resources of transplantable organs. Although the nonhuman primates would appear to be the ideal donor animals for human xenotransplantation because of both immunological and physiological similarities, there are several problems associated with their possible application: the majority of them belong to the group of endangered species, they are difficult to breed, their aseptic housing is not solved,

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[b] *Abbreviations*: CRP, complement regulatory protein; CsA, cyclosporine A; CVF, cobra venom factor; DAF, decay accelerating factor; DXR, delayed xenograft rejection; HAR, hyperacute rejection; MCP, membrane cofactor protein; sCR1, soluble complement receptor 1; Th, T-helper cell; XNA, xenoreactive antibodies.

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the techniques for their genetic manipulation have not yet been developed, and there are considerable infectious-microbiological and ethical concerns that do not favor their application. Therefore, the attention of the scientific world has turned toward the pig as a potential organ donor. The pig has several advantages: it is available in large numbers, easy to breed, it can be housed under aseptic conditions, the techniques for its genetic manipulation are known, and considering that it is consumed worldwide, the ethical concerns would be minor compared to that of nonhuman primates. Nevertheless, since the evolutionary distance between the swine and the human species is much greater than the corresponding distance between humans and the nonhuman primates, the immunological barriers appear to be more difficult to overcome in the pig to human xenotransplantation model than in the primate to human model [1-4].

Based on the experimental findings using pigs as donors and nonhuman primates, e.g., baboons, as recipients (in a few cases humans as well), a three-phase rejection process develops following the xenotransplantation of a solid, vascularized organ. Within minutes to hours after transplantation, the so called hyperacute rejection (HAR)[b] begins, which can destroy the organ in quite a short period of time. If HAR is somehow overcome, a slower rejection process, the delayed xenograft rejection (DXR, or acute vascular rejection) follows, which ruins the organ in several days. DXR is characterized by the progressive infiltration of the xenograft by natural killer cells and macrophages, the activation of endothelial cells, and by the marked aggregation of platelets. Xenoreactive antibodies (see below) that are of primary importance in the pathogenesis of HAR play central roles in the appearance of DXR as well. The relevance of DXR from a clinical point of view is related to the enormous doses of immunosuppressive medications needed to inhibit its progression [1-6]. Later, if somehow DXR could also be averted, the cell-mediated rejection becomes active. The cell-mediated rejection seems to bear the closest resemblance to the rejection processes of allotransplantation (i.e., the transplantation of organs between different individuals of the same species; this is the currently applied human transplantation technique) since it is associated with the structural differences of major histocompatibility (MHC) molecules between different species. As the transplanted organ must survive the processes of both HAR and DXR to develop cell-mediated rejection, the experimental knowledge about cell-mediated rejection is scarce when compared to HAR and DXR [1, 4-6]. The immunological mechanisms appear to be similar in the pig-to-human and pig-to-primate xenotransplantation settings, therefore, the pig-to-primate xenotranplantation is considered to be a good model for the pig-to-human xenotransplantation.

SYNOPSIS OF THE PROCESSES OF HYPERACUTE REJECTION

In the pathogenesis of HAR, the so called xenoreactive antibodies seem to be of primary significance. The majority of these xenoreactive antibodies (XNA) react with antigens of the porcine endothelium and lead to the destruction of the organ via the activation of the complement cascade. The main antigen recognized by these antibodies is the Galα1-3Galβ1-4GlcNAc (in the following Galα1-3Gal) oligosaccharide, which is abundantly expressed on the glycoproteins and glycolipids of porcine endothelial cells. (The antibodies recognizing Galα1-3Gal are termed anti-Gal in the following.) Unfortunately the XNAs naturally occur in high titers in humans, apes, and Old World monkeys (Catarrhines). XNAs (both IgG and IgM)

comprise 1 to 2 percent of the circulating immunoglobulins in humans [7].

HAR does not occur in the xenotransplantation models of all species combinations. The species combinations where HAR occurs (e.g., pig to human, pig to baboon) are called discordant, whereas those where HAR does not appear are termed concordant (e.g., mouse to rat, baboon to human). The difference between discordant and concordant species combinations is associated with the presence or absence of XNAs in the recipient's blood. The majority of animals studied to date express the Galα1-3Gal epitope on the endothelial cells, whereas humans and nonhuman primates do not [1, 5]. The absence of Galα1-3Gal expression in humans and primates is related to defects of the α1,3-galactosyl-transferase gene that catalyzes the assembly of the Galα1-3Gal molecule in other animals. Since similar galactosyl structures are found on bacteria (and other pathogens) as well, it is assumed that the appearance and high titer of these XNAs may be related to the more effective defense against certain pathogens, mainly bacteria [8]. The natural XNAs are absent at birth, but develop in a few weeks or months thereafter [7], possibly in association with the bacterial colonization of the gastrointestinal tract. Therefore the presence of XNAs is presumed to be advantageous for the immunity against bacteria, but deleterious in such an abnormal, "man-made" situation as the xenotransplantation. XNAs are supposed to be produced by B-cells of the $CD5^+$ subgroup [3, 9]. The production of these natural anti-Gal XNAs in humans does not seem to be suppressed by conventional immunosuppressive medications (e.g., Cyclosporine A [CsA], tacrolimus, azathioprine, mycophenolate mofetil, steroids) [10]. The XNAs responsible for HAR are of the IgM phenotype, which are very potent in activating the classical pathway of the complement cascade [7].

Although in the pig-to-primate (and pig-to-human) models the HAR seems to be primarily mediated via the classical pathway of complement activation, the alternative pathway also appears to contribute to the development of HAR mainly in rodent models (e.g., guinea pig to rat) [11]. The activation of the alternative pathway may be important in the ischemia-reperfusion injury of the xenograft in the pig to primate models [11].

The activation of the complement and the binding of XNAs result in the destruction of the endothelial cells [11, 12]. The XNAs lead to the activation of endothelial cells (Type 1 activation, i.e., without gene activation), the natural anticoagulants heparan sulphate and ecto ADP-ase are lost from the endothelial surface. These changes, together with the products of complement activation, may participate in the resulting microthrombus formation [4, 5]. The organ is finally destroyed by the consequent processes of interstitial oedema, thrombosis, and hemorrhage. The development of thrombosis seems to be associated with a shift of the coagulation system in the procoagulant direction: the efficacy of anticoagulant factors decreases, whereas the activity of procoagulant mediators increases. Molecular incompatibilities between different species may be important in this regard, since some anticoagulant factors do not work effectively across species barriers (e.g., porcine thrombomodulin does not effectively activate human protein C [13]; the porcine tissue factor pathway inhibitor does not adequately neutralize human factor Xa [14]), on the other hand the activity of certain procoagulant factors may even be enhanced (e.g., enhanced potential of porcine von Willebrand factor to associate with human platelet GPIb [15]). The coagulation and thrombotic disorders participate in the later phases of the rejection process as well, and may even manifest themselves in such systemic and life-

threatening forms as thrombotic thrombocytopenic microangiopathic state and disseminated intravascular coagulation [16].

Since the endothelium is the primary target of HAR, it is important to note, that tissues that do not have endogenous vessels, e.g., pancreatic islets are not prone to the destruction by HAR [17, 18].

The prolonged presence of a transplanted xenograft (if HAR could be averted) can enhance the titers of XNAs belonging to the IgG phenotype, that — on the other hand — seem to participate in the processes of the DXR. In addition to endothelial cells, these IgG anti-Gal antibodies also bind to epitopes on fibroblasts, chondrocytes, macrophages, basement membranes, and extracellular matrix [19]. The interaction of anti-Gal antibodies with these epitopes may participate in the development of a fibrotic response that is feared to present a severe chronic complication of xenograft rejection. The incubation of renal fibroblasts with anti-Gal antibodies resulted in increased transforming growth factor β and collagen synthesis [20]. The natural IgG anti-Gal antibodies appear to display polyreactivity, i.e. they were found to be reactive to DNA, actin, myosin, and tubulin, in addition to the Galα1-3Gal structures [21]. This finding may relate to a much broader physiological importance of these antibodies, since these natural, polyreactive antibodies are surmised to be important in maintaining immune homeostasis.

There are several possible ways to prevent HAR. These can be divided in two

Table 1. Summary of the techniques aimed at the prevention of hyperacute rejection.

Inhibiting the interaction of XNAs with the xenoantigens	
Recipient	**Donor**
1. Eliminating the XNAs from the recipient's circulation: plasmapheresis, immunoadsorption, perfusion via porcine organs (kidney, liver)	1. Infusion of α-galactosidase
2. Infusion of Gala1-3Gal oligosaccharides	2. Transfection of α-galactosidase
3. Anti-idiotype antibodies	3. Transfection of fucosyl-transferase
4. Accommodation (?)	
5. Suppression of XNA-producing B-cells	
6. Induction of tolerance (mixed chimerism, gene therapy)	
Inhibiting the activation of complement	
Recipient	**Donor**
1. Cobra venom factor	1. Transfection of human CRP (MCP, DCF, CD59)
2. C1-inhibitor	2. Transfection of porcine CRPs
3. Soluble complement receptor 1	

groups: methods influencing the recipients (i) and the donors (ii). In both groups, the main targets of intervention are the XNAs (and their antigens recognized) and the complement system. The treatment of the donors seems to be the safer and more convenient way. Table 1 summarizes the currently available strategies aimed at the prevention of HAR.

STRATEGIES TO TREAT THE RECIPIENT

The XNAs can be removed from the circulation by various methods, e.g., by plasmapheresis, perfusion via immunoaffinity columns or swine organs (e.g., liver, kidney). In a discordant pig to baboon heart transplantation model, HAR could be effectively prevented by an immunoabsorption technique [22]. The main problem related to these techniques is that they result only in a temporal diminution of XNA levels, and the antibody titers recur quickly. In patients who received ABO (blood group) incompatible transfusion, the phenomenon of accommodation was described, i.e. the temporary removal of the antibodies prevents HAR, and later, despite the return of the antibodies, no rejection occurs. Various mechanisms are supposed to participate in the genesis of accommodation, among these the masking of xenoantigens, changes in XNA affinity, or alterations in the gene expression of endothelial cells may be important. It may be surmised that a similar phenomenon may be useful in the prevention of HAR in xenotransplantation [7, 23]. *In vitro* data, however, do not unambiguously support this assumption.

In a rodent model of discordant xenotransplantation (transplantation of hamster hearts to presensitized rat recipients) a phenomenon similar to accommodation was observed. By using cobra venom factor (CVF) and CsA, HAR could be prevented, and later, by keeping anti-donor antibody levels low (by blood exchange, CVF, and CsA), HAR did not occur despite the later reappearance of XNAs. DXR, however, later evolved. The surviving hearts showed increased expression of protective genes (e.g., Bcl-2, Bcl-XL, A20, HO-1) in endothelial and smooth muscle cells, and a predominant intragraft Th2 response [24].

Another approach is to treat recipients with haptens like Galα1-3Gal, to inhibit the antigen-antibody reaction between the XNAs and the cellular antigens [25]. Anti-idiotype antibodies may also be applied to reduce the binding of XNAs [26]. The suppression of XNA-producing B-cell populations by anti-anti-Gal anti-idiotype antibodies is also being investigated [26].

The activity of the human complement system can be suppressed by various medications. CVF is (i) too toxic, because it leads to the generation of C3a and C5a, (ii) it is strongly antigenic, (iii) it loses its effectivity in a few days, (iv) it may lead to the elevation of anti-Gal antibody titers, probably in association with the presence of Gal epitopes in the structure of CVF [27]. The C1-inhibitor was found to be active in inhibiting the activation of the complement system in *in vitro* experimental models, especially when coadministered with heparin [28]. The application of soluble complement receptor 1 (sCR1) seems to be quite encouraging [29].

An alternative approach to prevent HAR (and later rejection processes) by treating the recipient appears to be the induction of tolerance mechanisms toward donor cells. By inducing tolerance mechanisms, long-lasting acceptance of the transplanted organs may be achieved. The production of anti-Gal antibodies can be inhibited if the immune cells (both T- and B-cells) of the recipient are made tolerant toward Galα1-3Gal epitopes [30, 31]. The most convenient way of tolerance induction is the generation of mixed chimerism by the introduction of donor hemopoietic

cells to the recipient. To achieve this, host conditioning regimens are needed to deplete donor-reactive host T-cells. Various protocols, e.g., total body irradiation, cytotoxic drugs, and antibodies against costimulatory molecules, can be applied to promote the successful implantation of donor stem cells. Since the naturally occurring XNAs would destroy infused xenogeneic bone marrow cells, the XNAs should be removed from the circulation prior to the infusion of the donor bone marrow [32]. By introducing donor bone marrow to the conditioned recipient the state of mixed chimerism can be achieved, where both donor and recipient hemopoietic cells can be found in the patient's bone marrow, and the newly developing T-cell repertoire is tolerant toward both donor and host cells. In a murine model of discordant xenotransplantation, by introducing Galα1-3Gal expressing donor cells to Galα1-3Gal knockout mice, the production of anti-Gal antibodies could be inhibited through the generation of mixed chimerism [33]. Galα1-3Gal knockout mice are homozygous for a targeted disruption of the α1,3-galactosyl-transferase gene, therefore they do not express the Galα1-3Gal epitope, and produce anti-Gal antibodies similar to primates [33]. Galα1-3Gal knockout mice harboring mixed chimerism were shown to accept Galα1-3Gal expressing heart xenografts [34].

The establishment of mixed chimerism in the pig-to-primate experimental system seems to be more problematic. Sablinski et al. managed to achieve long-term survival of pig bone marrow cells in cynomolgus monkeys by including pig cytokines (stem cell factor and interleukin-3) in the therapeutic regimen besides the immunosuppressive medication (CsA and 15-deoxyspergualin) [35]. Considering that the induction of tolerance is supposed to be related to the successful homing and differentiation of donor antigen-presenting cells in the recipient thymus to induce deletion of donor-reactive host cells, molecular incompatibilities between porcine and human adhesion molecules may be of primary significance. Studies performed to date examining the interactions between integrins, intercellular adhesion molecule-1 and CD44 showed that these molecules could interact with their ligands across the species barrier [36, 37]. It should be noted, however, that the introduction of animal bone marrow to a human recipient raises several questions including infectological and ethical problems, and the appearance of graft versus host disease (host tissue destruction by donor immune cells) cannot be excluded either.

To avoid the need for animal bone marrow cells, investigations are also aimed at the inhibition of XNA production by gene therapy. By introducing the enzyme α1,3-galactosyl-transferase to autologous bone marrow cells of Galα1-3Gal knockout mice by retroviral gene therapy, Galα1-3Gal epitopes become expressed on transfected cells. The expression of Galα1-3Gal epitopes rendered the mice tolerant toward these antigens and the production of anti-Gal XNAs ceased [38]. The presence of the transfected gene could be regarded as molecular chimerism. By this protocol the need for xenogeneic bone marrow transplantation could be avoided. However, it should also be taken into account that in contrast to the whole bone marrow transplantation only a few antigens can be introduced by the gene therapy, therefore the individual would only be rendered tolerant to a minority of antigens relevant in rejection processes.

STRATEGIES TO TREAT THE DONOR

It should be noted that recent data show that HAR does not invariably appear

in discordant xenotransplantation models. By increasing the donor organ size and weight (in a kidney transplantation model) the frequency of HAR could be reduced. This phenomenon may be linked to the immunoabsorption of the preformed antibodies by the larger grafts without significant damage to the organ as a whole [39].

A great advantage of xenotransplantation versus allotransplantation can be the fact that the donor of xenotransplantation is known long before the operation and, therefore, it can be modulated to promote the successful implantation of the graft. Molecular biological techniques are known for the genetic manipulation of pigs, and several approaches are being tried to prevent HAR in the recipient. Considering that the techniques for cloning pigs from somatic cells have only been described recently [40, 41], and successful gene knockout experiments in pigs have not yet been performed, up to now indirect methods were used for the diminution or elimination of the anti-Galα1-3Gal response. The enzyme that degrades Galα1-3Gal, α-galactosidase can be transfected to pigs. A more promising approach is the transfection of alpha-1,2-fucosyl-transferase that uses the same substrate as galactosyl-transferase (i.e., N-acetyllactosamine), so by an elevated level of fucosyl-transferase the expression of Galα1-3Gal can be reduced, due to a competitive mechanism [26, 42].

The Galα1-3Gal epitopes can also be removed by enzymatic treatment, but this appears to be a temporary solution, because following their removal, the molecules will be soon regenerated [43]. Nevertheless the enzymatic treatment of pig organs shortly before transplantation can be regarded as a useful subsidiary treatment.

Another possible approach to prevent HAR is based upon the phenomenon of homologous restriction of complement activation. It is known for decades that the complement0-mediated cytolysis is not efficient when the complement and the target cells are of the same species, a phenomenon called homologous restriction [7]. Recent investigations shed light on the mechanism of homologous restriction. It turned out that the protein inhibitors of complement activation (complement regulatory proteins: MCP [membrane cofactor protein, CD46], DAF [decay acceleration factor, CD55], and CD59) act in a species-specific manner. Therefore, the theory emerged that by introducing human complement regulatory proteins (CRP) to swine, HAR can be inhibited. Indeed, by generating transgenic pigs for human CD59 and DAF, HAR can be prevented in baboons transplanted with these modified pig organs [12, 44, 45]. The inhibition is more effective if multiple transgenes are present. Nevertheless, these organs are still susceptible to DXR, which eventually leads to their destruction. In cynomolgus monkeys transplanted with transgenic porcine organs transfected with human DAF, survival of nearly 80 days was observed by applying a combined immunosuppressive strategy (splenectomy, CsA, cyclophosphamide, steroids) [46].

The complement-inhibiting properties of MCP and DAF can be combined in a chimeric recombinant protein that turned out to be effective in an *in vitro* model of pig to human heart transplantation [47].

Recent data, however, cast doubt on the unambiguous validity of homologous restriction. Several research groups found that swine CRPs can efficiently inhibit human complement activation. MCP [40] and CD59 [49-51] inhibited the activation of the human complement cascade in a similar extent as their human counterparts did. By up-regulating CD59 expression on porcine endothelial cells by treatment with a Galα1-3Gal binding lectin, the cells could be made resistant to the deleterious effects of human complement activation

[52]. Therefore it can be surmised that not the human CRPs themselves but their overexpression in the modified organs is responsible for the inhibition of human complement activation, and it is possible that the overexpression of swine CRPs would be equally effective.

PROBLEMS ASSOCIATED WITH THE TECHNIQUES AIMED AT THE PREVENTION OF HAR

A major problem with the elimination of anti-Galα1-3Gal antibodies in the recipient and Galα1-3Gal epitopes of the donor is associated with the possible protective role of the antibodies against certain infections. Not only bacteria, but also some viruses of animal origin bear Galα1-3Gal epitopes in their membranes (that derive from the infected cells of the animal host). The anti-Gal XNAs are supposed to participate in the complement-mediated lysis of enveloped viruses. If the graft does not express Galα1-3Gal, the viruses that bud off from the cellular membranes of such modified organs will also lack this antigen, thus the XNAs would be ineffective against them [2, 8, 53].

One of the most important possible dangers linked to xenotransplantation is considered to be the infectious concern. Among the infectious agents, viruses are the most feared [2, 54]. At least 1 percent of the mammalian genome is composed of endogenous retroviruses [55]. The majority of these is presumed to be defective, but there are experimental data that primate, and unfortunately porcine endogenous retroviruses as well, may infect human cells [56].

Although Galα1-3Gal is thought to be the major xenoantigen in HAR, other antigens may also be important, and these could have pronounced roles in the rejection processes if Galα1-3Gal has been eliminated [57]. There are some data in rodent models concerning the importance of these alternative antigens. Removal of Galα1-3Gal epitopes in mice by galactosyltransferase knockout and fucosyltransferase transfection techniques leads to the presentation of additional epitopes (e.g., the Forsmann antigen) that may also induce a xenogeneic immune response [42]. A further question concerns the swine endothelium: by reducing the expression of Galα1-3Gal, other, previously hidden antigens will be exposed that could lead to further complications. It can also be supposed that the Galα1-3Gal molecules may be important in the functioning of the pig endothel, and by removing them, we could interfere with the normal development of pig organs.

Since some human CRPs are known to be the receptors of viruses, e.g., MCP is the receptor for measles virus [58], DAF for ECHO and Coxsackie viruses [59-61], the transfection of human CRPs may render pig organs susceptible to human viral infections. The infection of CD46 transfected pigs with measles virus could interfere with the cellular differentiation of the organs. Recent investigations have shown that the CRPs may also be important in the activation of immune cells, and by introducing human CRPs to pigs the inhibition of HAR may be followed by enhanced cellular rejection mechanisms. DAF is the ligand of CD97 that is expressed on activated leukocytes [62]. Human CD59 was found to exert costimulatory activity on human T cells acting through CD2 [63]. By using porcine CRPs these problems could be averted. In addition to the use of porcine CRPs, other methods may also be applied to circumvent the problem of graft virus susceptibility. In the case of CD46, by molecular remodeling techniques, CD46 variants without measles receptor activity are being developed that at the same time seem to be even more potent than wild-type CD46 in inhibiting human complement activation [64]. A further problem may be related to the possibility

that by overexpressing CRPs (either human or porcine) the enveloped viruses released from the transfected porcine organs may carry these CRPs in their envelopes, thereby, they may be rendered resistant to the attack of human complement [63].

A serious concern may be related to the physiology of the transgenic animals. Will the organs (and the animals themselves) differentiate and function properly, as their wild-type counterparts? This concern may be most pronounced in association with the techniques that reduce the Galα1-3Gal expression. In fact, mice with inactivated galactosyl-transferase genes appear to develop normally, but develop eye cataracts [65].

CONCLUDING REMARKS

Significant progress has been made in recent years concerning the understanding of the molecular mechanisms underlying the pathogenesis of hyperacute rejection. Numerous methods can be potentially useful to avert this formidable barrier to xenotransplantation. Among these, the techniques applying the genetic modification of the donor animals appear to be the most promising. The recent success of pig cloning could contribute to considerable advances in the investigation of xenotransplantation. Nevertheless, serious problems may be associated with some of these methods. The infectious concern seems to be the most significant. Careful investigations are required before these techniques may enter the field of clinical medicine.

It should be noted that by averting HAR, further immunological barriers appear. Among these delayed xenograft rejection (DXR) seems to be the most important, since unacceptably high levels of chronic nonspecific immunosuppression would be needed to inhibit its progression. XNAs play central roles in the pathogenesis of DXR as well, but the described techniques that seem to be appropriate for the elimination of HAR do not appear to be sufficient for the prevention of DXR. Since the prevention of HAR can be considered as possible, at present, DXR seems to present the most formidable obstacle in the way of successful xenotransplantation. α1,3-galactosyltransferase knockout pigs could be the solution for the prevention of both HAR and DXR. Other problems (e.g., physiological) may also severely impede the application of xenotransplantation to solve the current organ shortage.

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