XENOGENEIC LIVER TRANSPLANTATION

Potential applications and pitfalls in a discordant rodent model

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Xenogeneic liver transplantation

Potential applications and pitfalls
in a discordant rodent model

Xenogene lever transplantatie

Potentiële toepassingen en valkuilen
in een discordant knaagdier model

PROEFSCHRIFT

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aan de Erasmus Universiteit Rotterdam
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Nog zoveel toekomstdromen ...

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Introduction

Introduction to xenotransplantation

After the initial focus on the biology of graft rejection and its prevention, now the main concern of transplantation research is donor shortage. In 1979, the introduction of cyclosporine A (CsA) improved the outcome of clinical organ transplantation significantly. Technical developments and experience further increased survival rates. Therefore, serious efforts have been made to raise the amount of potential donors. These efforts included public awareness campaigns, altered donation legislation and expanded clinical donation programs [1,2]. Despite these initiatives, there was no reduction in the number of patients awaiting transplantation. Thus, interest has focussed on technical and scientific advances to broaden the possibilities of mastering this problem. The development of artificial organs and progress in cloning techniques might lead to a solution. Xenotransplantation, transplantation of organs and tissues from one species to another, including man, also holds a high potential. It could offer the opportunity of access to an indefinite pool of donors.

History

The use of animals to overcome human derived problems is not new. In myths, minotaurs and centaurs are common creatures, whereas Daedelus was probably the first with a functioning animal graft of feathers. Like in 'allotransplantation' the Greek prefix 'xeno', meaning 'foreign', in xenotransplantation describes the relationship between graft and host.

In 1905, serious transplantation efforts were performed in animal to human grafting. Princeteau used rabbit kidney slices to overcome renal insufficiency [3]. One year later, Jaboulay tried to graft pig and goat kidney but they remained without transplant function [4]. In 1905, non-human primate to human renal transplantation resulted in a survival of 32 hours [5]. In 1923, a patient lived for 9 days with a grafted lamb kidney [6]. In the 1960s, various groups worked on kidney xenotransplantation due to the absence of donor organs and the lacked availability of chronic dialysis. However, most recipients were unable to return to normal life or even survive more than a few months [7]. Xenotransplantation was abandoned after the development of successful cadaver organ procurement programs and the introduction of immunosuppressants, which controlled allogeneic but not xenogeneic rejection. During the 1980s, renewed interest grew because of the exponential increase in donor organ shortage. In 1985, 'Baby Fae' received a baboon heart but failed to survive for more than 3 weeks [8]. In 1992, two patients received baboon livers and survived 26 and 70 days, respectively [9,10].

Concordant and discordant

Calne coined concordant and discordant terminology in xenotransplantation to identify and describe the type of rejection between closely related (concordant) and widely disparate species (discordant) [11]. Transplantation between concordant species was thought to result in acute cellular rejection, whereas antibody-mediated rejection occurred in discordant combinations. Increased knowledge led to the understanding that antibodies can play a major role in both kinds of rejection. Consequently, the terms were only used to describe the phylogenetic distance between two species. In most cases this coincides with their rejection pattern. Concordant rejection takes place after a few days. In general, antibodies are formed after transplantation that reject a graft in an antibody-mediated and an antibody-dependent cellular way. Examples of concordant combinations are hamster to rat, goat to sheep, vervet monkey to baboon and non-human primate to human. In discordant rejection, preformed xenoreactive natural antibodies together with complement destroy a graft within minutes to hours after transplantation. Discordant combinations include guinea pig to rat, pig to dog and pig to human.

Future species for organ donation

In human allogeneic organ grafting, organ availability is one of the major issues. Donor MHC, blood group and organ size should match the recipient as close as possible. In xenotransplantation, anatomical and immunological differences are even more important than in allotransplantation. Xenograft survival seems to be inversely proportional to the phylogenetic distance between the two species [12]. Transplantation between two species of one zoological family (e.g. between primate and man) usually results in acute cellular rejection, whereas between two families within an order (e.g. between monkey and man) a mixed humoral and accelerated cellular rejection follows. When the barrier of zoological orders is crossed, hyperacute rejection is seen, which is dominated by antibody-mediated mechanisms. However, many inconsistencies lay within this general pattern [13].

Primates and monkeys were first used as donors in clinical transplantation [9,14]. Although this model showed promising results, it will never offer the solution for donor shortage (see Table 1). The most important factor for not using primates is that primate reproduction for transplantation is considered unethical. The use of more distantly related 'farming' animals, like pig, sheep or cow, is less hampered by these problems. The pig is currently the most likely species to be considered for future xenotransplantation purposes (see Table 1).

Table 1. *Advantages vs. disadvantages for the use of primate and pig donors in clinical xenotransplantation.*

Donor species	Disadvantage	Advantage
Primate	- low reproduction rate [9] - relatively few primates of suitable size - of all primates only chimpanzees have blood group O - great risk of transferring specific pathogens to human [15] - unethical	- relatively simple rejection processes - similar physiology
Pig	- complex immunological rejection processes - disparate physiology, although some similarities [16,17] - potential damage of pathogen transfer [18,19]	- less ethical problems - suitable size - rapid breeding, large litters - knowledge about pig genetics - transgenesis

To study xenotransplantation in an easier and faster way, small animal models have been developed with mainly the rat as organ recipient. The immunology of the rat has been subject of research for many years and has been characterised in detail. The process of rejection in particular reveals similarities between rat and human. Organ donors, like mouse, hamster or guinea pig, correspond to primates, monkeys and pigs, respectively for the human situation. In this way, clinical hypotheses can be tested promptly in relevant animal models.

Xenograft rejection processes

Like in infectious processes, antibody formation, complement activation and cellular immunity play major roles in transplantation ultimately leading to 'rejection'. In xenotransplantation, the rejection of grafts varies from minutes to more than a week. This difference in outcome severely depends on the donor-host combination. In concordant grafting, acute rejection takes places after a few days to a week and is partially comparable to allograft rejection. Discordant grafting, however, reveals a totally different scheme. The processes seen here, in order of appearance, are hyperacute rejection (HAR), delayed xenograft rejection (DXR) or acute vascular rejection (AVR) and possibly a T-cell mediated rejection (see Figure 1). The first two processes are

unique in xenotransplantation and are caused by a few well-defined distinguishing details when compared to allotransplantation.

Concordant rejection process

Although comparable to allograft rejection, concordant xenograft rejection presents specific characteristics. The hamster to rat model is frequently used to study this type of rejection in which grafts are rejected over several days [21]. To extend graft survival, a wide variety of immunosuppressive regimens is successfully used [22-24]. In general, protocols combining both anti-humoral and anti-T-cell immunosuppression are most effective. Extended hamster xenograft survival seems to require prolonged suppression of rat xenoreactive antibody synthesis or prevention of the deleterious consequences of anti-donor antibody binding to the graft, or both. The translation, however, of the rodent model to the clinical situation bears some inconsistencies. In the concordant cynomolgus monkey to baboon model prolonged survival was found after treatment with CsA, steroids, azathioprine and ATG [25]. The eventual graft loss correlated with an increase in cytotoxic antibodies [25]. No prolongation was seen in the vervet monkey to baboon model after treatment with conventional immunosuppression [26,27]. Also in the baboon to human situation, grafted livers displayed evidence of antibody-mediated injury post-transplant [9]. This indicates that the acquired antibody response is stronger than in allografting and that conventional immunosuppression is not sufficient to suppress this mechanism of the immune response.

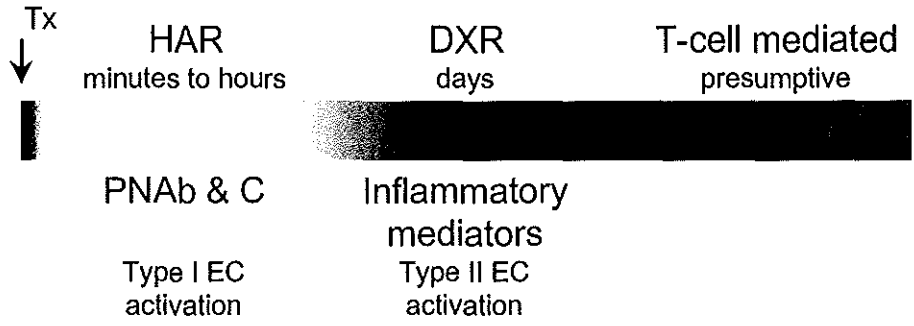

Figure 1. *Phases of rejection of discordant vascularised grafts.*
The bar indicates the chronological course of the different subsequent phases of discordant graft rejection, i.e. hyperacute rejection (HAR), delayed xenograft rejection (DXR) and T-cell mediated rejection. Mediators of HAR are preformed natural antibodies (PNAb) and complement (C). Inflammatory mediators presumably initiate DXR. Moreover, the phases are not strictly separated but influence each other. The grey-scale indicates the present status of knowledge. The darker, the less informed we are about mechanisms of action in discordant grafting. (adapted from Bach et al. [20])

Hyperacute rejection – Discordant rejection processes

Xenograft transplantation between discordant species results in a very fierce process. Within minutes to hours, the organ is rejected [28]. Immediately upon reperfusion, interstitial haemorrhage and diffuse thrombosis starts. Internal organ structures break down. This process resembles the reaction seen after ABO-mismatched allograft transplantation.

HAR is based on two primary factors: 1. binding of the host preformed xenoreactive natural antibodies (PNAb) to antigens on endothelial cells in the graft, and 2. the incompatibility of complement regulatory proteins of the graft to regulate the complement system of the host, leading to uncontrolled complement activation [29,30]. The influence of each factor on hyperacute rejection depends on the model used.

Preformed xenoreactive natural antibodies

The immediate destruction of grafts correlates with the presence of donor specific antibodies before transplantation [31]. In case of allotransplantation such antibodies are the result of prior exposure to alloantigens. In xenotransplantation immediate destruction of grafts is observed in many cases without an acquired antibody response. More specifically, in discordant transplantation, antibodies against donor tissue circulate without any known history of sensitisation. Platt et al. demonstrated that grafts after HAR contained host antibodies that are co-deposited by components of the classical pathway of complement activation [32]. Already in the early 70's, Moberg et al. indicated that removal of preformed antibodies could lead to prolonged graft survival [33], which was confirmed by Cooper et al. [34].

The presence or absence of PNAb is more relative than absolute [35]. Concentration rather than presence of PNAb seems to affect the final outcome [35]. All mammals have been found to possess PNAb [36]. The best known examples of PNAb are the anti-ABO blood group antibodies.

It has been demonstrated that PNAb recognise a specific sugar epitope on endothelial cells [37]. It was first suggested by Good et al. that these antibodies might bind to Gal-α1,3-Gal [37]. The elution of antibodies from human-plasma-perfused porcine organs was indeed capable of binding terminal α-galactose residues on carbohydrates [38]. During evolution, differences between anatomy and appearance of species came into existence. Also genetic make-up and, therefore, cellular epitopes became different between species. It was found that humans, primates, Old World monkeys and some other species (ostriches and alligators) do without the sugar epitope Gal-α1,3-Gal [39]. As a consequence of evolution, PNAb reacted with these specific endothelial cell glycoproteins [40]. Pre-incubation with the specific glycoproteins was able to block about 90% of the binding to endothelial cells [40]. However, it can not be excluded that

PNAb recognise other structures as pig, goat, dog, rat and others can reject grafts hyperacutely but do not posses anti-Gal-α1,3-Gal antibodies [41,42].
These antibodies are predominantly IgM [43], which means that after removal they might not come back. Also IgG and IgA subclasses were found [44,45].
The origin and presence of these PNAb is subject to discussion. One possibility is that they are transferred from mother to child via placenta, colostrum or milk [36]. Galili speculated that evolutionary pressure, possibly infections by pathogens containing α-galactosyl epitopes, caused inactivation of the gene for α1,3-galactosyl transferase [39]. Therefore, the production of α-galactosyl epitopes was prevented [39]. A third and provocative hypothesis is that they are produced by distinctive B-1 B-cells, in contrast to conventional B-2 B-cells that react to acquired immune responses [46]. Both marrow-derived and peritoneal B-cells can efficiently produce PNAb [47]. The last possibility is that infections by environmental pathogenic agents lead to the formation of antibodies that cross-react with xenografted tissue.

Complement cascade

The complement system is a complex cascade as part of the innate immune system that plays a key role in the host defence process. Multiple plasma and membrane proteins, which now number more than 30 proteins, are part of this system. Complement has two major functions. 1. Modifications of membranes: Foreign surfaces, including grafted organs, are recognised and the cascade is activated. This leads to the disruption of the membrane integrity (lysis) and marks the surface for elimination (opsonisation). 2. Promotion of inflammatory response: During complement activation, small complement fragments diffuse away leading to directed cellular movement (chemotaxis) and promoting release of mediators (e.g. histamine) by binding to complement receptors (anaphylaxis). Therefore, it serves as a recognition and effector system of humoral immunity.
The discovery of the heat-labile activity present in normal serum was first described by Bordet and Gengou [48]. Ehrlich was the first one who applied the term "complement" to describe the activity in serum which "complemented" the ability of specific antibodies to cause lysis of bacteria [49]. The idea that complement not only assists antibodies as an effector of the humoral system, but that it also functions as an independent immune system was described by Pillemer et al. [50]. Because of the self-amplying, proinflammatory and destructive capabilities of complement factors, nearly half of them act as regulatory proteins and are capable in distinguishing between self and non-self [51].

Complement pathways

Two activation routes have been characterised. They are differently triggered, but merge at a critical step involving C3 (see Figure 2). The classical pathway is activated when

antibodies bind to antigens. The antibody, therefore, defines the target. The binding of complement initiates a series of reactions involving C1, C4, C2 and C3, resulting in the C3-convertase ***C4b2a*** and C5-convertase ***C4b2a3b*** [52]. Complement assists antibodies as an efficient effector of the humoral immune system. Between host and pathogen (donor), no prior contact is necessary to trigger the alternative pathway. A constant activation, "tick-over", of an activated complement component drives this route [53,54]. Its components, C3, factor B and P, bind and amplify quickly only in the presence of foreign material [55] and results in the formation of the C3-convertase ***C3bBbP*** and C5-convertase ***$C3b_nBbP$*** [56]. The final part of the cascade, the terminal complement complex (TCC) pathway, contains the components C5, C6, C7, C8 and C9. Together they form transmembrane pores, which are regarded as lethal by giving rise to osmolytic influx.

In xenotransplantation, complement activation leads to the disruption of the integrity of the blood vessels, extravasation, inflammation, endothelial cell activation and finally thrombus formation.

Complement regulation

Because of the destructive and proinflammatory capabilities of complement, control mechanisms are necessary [57]. These mechanisms limit the time of activation state of complement components to avoid excessive use of these components and to restrict the process. Therefore, the control is achieved by different mechanisms. Short half-lives are ensured by the fast spontaneous decay of activated components. Secondly, specific proteins proteolytically assist the inactivation of components. Both fluid-phase and membrane-bound regulation is needed. An overview of complement regulators is presented in Table 2. All mammals regulate complement activation to distinguish between self and non-self [51]. Therefore, transplanted organs should be able to control complement activation. However, in xenotransplantation, especially in discordant grafting, massive complement activation is seen. This is due to the fact that donor regulators of complement are ineffective in controlling host complement activation, as was first suggested by Platt et al. [30]. Early studies to test this hypothesis demonstrated that pig endothelial cells expressing human decay accelerating factor in their membranes were protected from lysis by human serum [58,59]. This indicated that porcine complement regulatory proteins are unable to control human complement activation, leading to massive complement-mediated damage. Other studies reaffirmed these findings [60,61]. Current studies mainly focus on transgenesis of membrane-bound complement regulatory proteins [62-64].

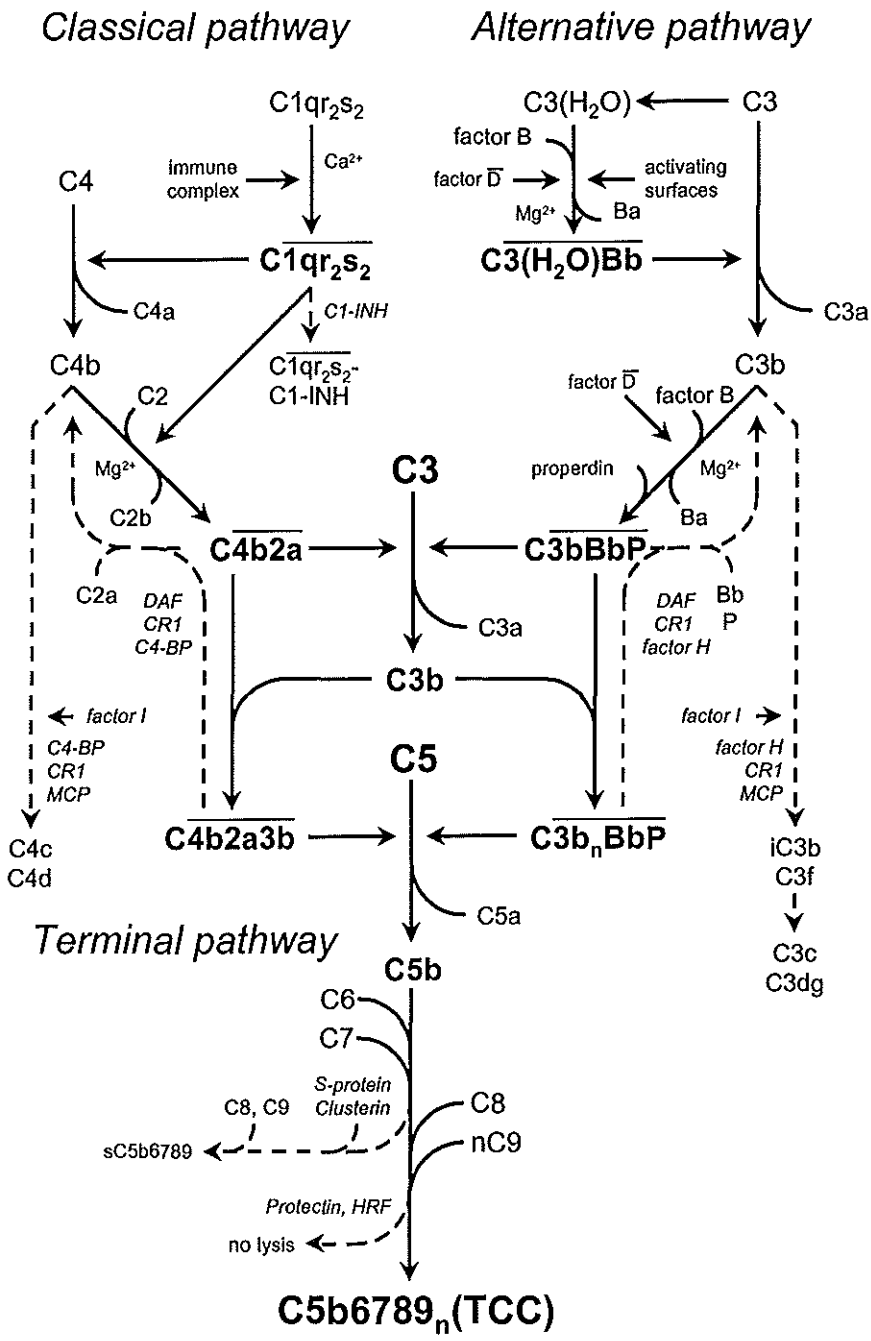

Figure 2. *The complement cascade with the classical and alternative activation pathways.*

Dotted lines depict the routes of complement regulator activation. Complement regulators are indicated in italics and enzymatically active components denoted by a horizontal bar.

Table 2. Regulators of complement

Complement regulator	Pathway[a]	Location	Function
C1-inhibitor (C1-INH)	classical	fluid-phase	inhibits activation and complexes C1 [65,66]
Properdin	alternative	fluid-phase	stabilises C3/C5 convertase [67,68]
Factor I	both	fluid-phase	degrades C3b/C4b, but requires co-factors [69-73]
Membrane co-factor protein (MCP, CD46)	both	membrane	binds C3b/C4b [73]
C4-binding protein (C4-BP)	classical	fluid-phase	binds C4b, inhibits formation and accelerates decay of C3/C5 convertase [69,74,75]
Factor H	alternative	fluid-phase	binds C3b, inhibits formation and accelerates decay of C3/C5 convertase [71,76]
Complement receptor 1 (CR1)	both	fluid-phase	binds C3b/C4b, accelerates decay of C3/C5 convertase [70,72,77,78]
Decay accelerating factor (DAF, CD55)	both	membrane	accelerates decay of C3/C5 convertase [29,79]
S-protein	terminal	fluid-phase	binds C5b-7 [80]
Clusterin (SP-40,40)	terminal	fluid-phase	binds C5b-7 [81]
Homologous restriction factor (HRF) or C8-binding protein (C8-BP)	terminal	membrane	inhibits perforation [82]
Protectin (CD59)	terminal	membrane	inhibits insertion of C9 [83]

[a]both: classical and alternative.

Anaphylatoxins

During activation of complement the small cleavage products C3a, C4a and C5a are generated. These anaphylatoxins are released in the fluid phase and bind to receptors on circulating blood cells (except erythrocytes) and some tissue cells.

C5a is the most powerful anaphylatoxin followed by C3a and distantly by C4a. The C5a-receptor is distinct from the C3a/C4a receptor, although they are often expressed on the same cell types such as monocytes, macrophages, granulocytes, endothelial cells and platelets. After binding of the anaphylatoxins, histamine and other mediators are released and cause vasoconstriction and increased vascular permeability. Additionally, C5a is a potent chemotactic factor and induces the directed migration of leukocytes to the site of inflammation [84]. It may also give rise to the release of IL-1α, TNFα and IL8 from monocytes [85].

Endothelial cell activation

As depicted above, PNAb and complement can induce total destruction of discordant grafted tissue. The underlying process involves mainly endothelial cell (EC) activation. EC form a physical barrier to keep cells and proteins in the intravascular space and prevent leakage into the extracellular fluids. It also promotes an anticoagulant environment.

Two types of EC activation are known [86]. Type I activation involves retraction of microvascular EC, exposing the subendothelial matrix. P-selectin and von Willebrand factor are expressed and platelet activating factor is secreted together with other inflammatory mediators, eventually leading to thrombosis. This type of activation is protein synthesis independent. Type II activation includes progressive induction of gene transcription expressing leukocyte adhesion molecules (E-/P-selectin, ICAM-1, VCAM-1), tissue factor and cytokines.

Bach et al. proposed a model of type I EC activation and HAR [20] (see Figure 3).

Therapeutic strategies to eleminate PNAb and their epitopes

To block the effect of PNAb on xenograft rejection various techniques have been used. Firstly, plasmapheresis was proven useful in clinically ABO-mismatched kidneys [89]. Application of this method in xenotransplantation, with or without immunosuppression, also resulted in prolonged survival of the graft [90,91].

A more elegant method is the use of extracorporeal absorption columns [92,93]. Protein A and G or anti-IgG and IgM antibodies effectively remove IgG and IgM, respectively [94,95]. However, it does not distinguish between natural and other antibodies. Extracorporeal organ perfusion effectively eliminates PNAb only [96]. Yet, this technique bears several disadvantages such as experimental intricacy, sequestration of blood volume and activation of complement and coagulation pathways.

A third possibility that might reduce the effect of PNAb is the use of specially designed peptides that mimic the α1,3-Gal determinant on xenografts [97,98]. Direct infusion of such peptides might be able to bind PNAb, thereby preventing them from activating EC. It has already been demonstrated that such a peptide could inhibit the agglutination of pig red blood cells by human serum at concentrations similar to that of Gal-α1,3-Gal [99].

The use of depleting antibodies, like anti-IgM monoclonal antibodies, was able to maintain low levels of not only total IgM but also xenoreactive antibodies [100,101].

The elimination of PNAb targets can lastly be mastered by genetic engineering of donor animals. The carbohydrate Gal-α1,3-Gal is synthesised by α1,3-galactosyl transferase. By 'knocking out' this gene (Gal KO) no target will be available for PNAb. For future application more targets might have to be addressed [102-104]. Animals expressing human α1,2-fucosyl transferase (H-transferase) with or without Gal KO have been

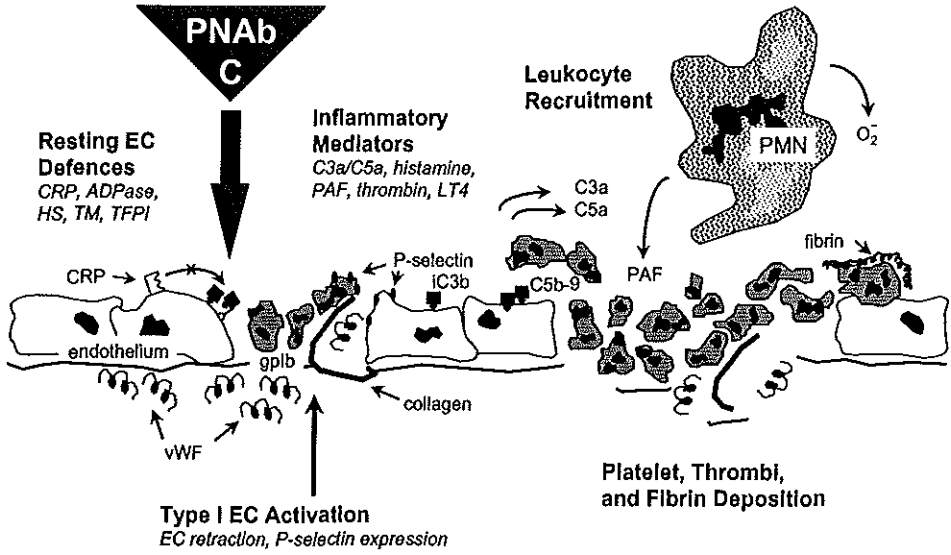

Figure 3. *Model of type I endothelial cell activation and hyperacute rejection.* Quiescent endothelial cells express different defence mechanisms that maintain the barrier between the intravascular space and organ parenchyma. Coagulation is inhibited by thrombomodulin (TM), tissue factor pathway inhibitor (TFPI) and ADPase, which inhibits platelet plug formation. Heparan sulfate (HS) is present in cell membranes and extracellular matrix of blood vessels and binds anti-thrombin III, a very powerful anticoagulant [87]. HS also binds superoxide dismutase that degrades oxygen radicals [88]. Endothelial cells have complement regulatory proteins (CRP) as well. Complement (C) activation with or without help from preformed natural antibodies (PNAb) is not regulated by CRP in a discordant setting [30]. Endothelial cell retraction leads to oedema and haemorrhage (not shown). Interaction between platelet receptor gpIb and von Willebrand factor (vWF) results in platelet adherence. Platelet activation is accompanied by release of inflammatory mediators including complement, platelet activating factor (PAF), thrombin and leukotrienes. This leads to polymorphonuclear granulocyte (PMN) attraction and further recruitment of platelets, resulting in promotion of coagulation and fibrin deposition. (adapted from Bach et al. [20])

generated [105-107]. H-transferase expression reduced the number of α-Gal epitopes in porcine and murine tissue [105,106]. Human plasma perfused hearts from H-transferase, Gal KO and H-transferase/Gal KO mice demonstrated prolonged survival as compared to their wild type controls [108].

Therapeutic strategies to prevent complement activation

Precluding the activation of complement has been proven extremely effective for mastering HAR. So far, cobra venom factor (CVF) is the most potent agent known. In the 1960s, cobra venom played a key role in the elucidation of alternative pathway

activation [109]. From this venom a special factor, CVF, was purified, which activates the complement system [110]. CVF was revealed as a functional analogue of C3b with many structural similarities [111,112]. Like C3b, CVF binds factor B. The CVF-Bb complex can function as a C3-convertase and when obtained from the Thai cobra (*naja naja kaouthia*) also as a C5-convertase. In contrast to the C3/C5-convertases, CVF-Bb is rather stable with a $t_{½}$ of 7 hr compared to 1.5 hr for ***C3bBb*** [67,113]. Complete resistance is seen to decay acceleration by factor H and proteolytic inactivation by factor I [54,114]. Therefore, the administration of CVF leads to continuous complement activation until one or more complement components are depleted. In discordant grafting, CVF prolongs survival times from minutes to hours [115-118]. However, clinical use may be limited due to its toxicity as well as immunogenicity.

Soluble complement receptor 1 (sCR1) inhibits complement activation and, therefore, does not lead to depletion. sCR1 accelerates the proteolytic cleavage of C3/C5-convertases of both pathways, thereby preventing the development of TCC, anaphylatoxins and cell adhesion. The effectiveness of sCR1 in xenotransplantation is evidenced by its capacity to prolong survival times from minutes to several hours [119-121]. Several other substances, including peptides that mimic or block complement components, have been tested but were found to be less effective or are still experimental [122,123].

As depicted in Figure 2, complement activation is continuously regulated by complement regulatory proteins like DAF, MCP or CD59, which are located on most cells. Species specificity prevents the down-regulation of complement activation upon xenotransplantation [30]. Porcine tissue transgenic for human complement regulatory proteins is protected from cytotoxic effects of human complement [59,124]. Genetic engineered mice and pigs show very promising results in *ex vivo* perfusion models as well as *in vivo* experiments [62,125-130]. Some complement regulatory proteins are more effective than others as was demonstrated by Marquet et al. [63]. Even animals, transgenic for two complement regulatory proteins have been created [131,132]. Additive effects were obtained by combining DAF (and CD59) with GalKO or H-transferase in transgenic mouse hearts perfused with human serum [132,133].

Therapeutic strategies to prevent coagulation

Besides targeting the PNAb, its epitopes and complement activation, other strategies focus on thromboregulation. Administration of drugs that prevent coagulation prolonged survival of discordant grafts [134,135], but strong complement activation still occurs leading to HAR [135,136]. These experiments suggest an important role for thromboregulatory mechanisms in HAR. However, targeting EC activation as the main cause of disordered thromboregulation seems more reasonable.

Delayed xenograft rejection – Discordant rejection processes

In discordant models, preventing HAR by controlling complement activation prolongs graft survival. Still after a few days, rejection occurs in a process called delayed xenograft rejection (DXR) [137] or acute vascular rejection [138]. Cellular infiltrates appear to play a pivotal role in DXR in contrast to HAR [139]. Low number of lymphocytes concur with infiltrating NK-cells, monocytes and macrophages [137,139-141]. The influence of these cellular infiltrates is not yet clear [142]. The onset of DXR coincides with a rise in antibody synthesis [143]. Removal of PNAb from a xenograft recipient delays the onset of rejection and suppression of antibody synthesis prolongs survival even more. [138,144]. Therefore, antibodies still may be the main initiators of EC and monocyte activation, although DXR also occurs in the absence of PNAb [20,145]. Deposition of early components of the classical pathway can lead to changes associated with DXR [146]. Monocytes, even in a complement depleted or regulated environment, are a major source of extrahepatic complement synthesis [147,148].

Endothelial cell activation

Bach et al. proposed a model of type II EC activation and DXR [20] (see Figure 4). Activated monocytes and its cytokines probably induce this activation [149-151]. It includes progressive induction of gene transcription expressing leukocyte adhesion molecules (E-/P-selectin, ICAM-1, VCAM-1) and tissue factor. Thrombin can potentiate endothelial cell activation via NFκB [152]. The fact that this reaction is stronger in discordant grafting than in allotransplantation is at least partly due to molecular incompatibilities [153-155]. Moreover, traditional immunosuppressive treatments, which primarily inhibit lymphocyte proliferation, are ineffective in xenografting. Only extreme immunosuppression leads to xenograft survival up to 90 days [125].

Therapeutic strategies

As already stated earlier, traditional immunosuppressants are not very powerful in overcoming xenograft rejection. Non-specific immunosuppressive drugs are only successful in preventing the formation of induced antibodies [159]. Experiments have been carried out to inhibit platelet activation with marginal results [160]. Although other substances are under investigation, rejection is only postponed [161]. Inhibition of B-cell responses seems to be the most effective [161,162]. Anti-coagulant therapy in a complement deficient environment prolonged discordant graft survival showing decreased platelet aggregation and inflammatory infiltrates [163].

Other strategies concentrate on genetic manipulation. Upregulation of transcription factor NFκB leads to gene transcription of molecules involved in DXR [164]. Overexpression of IκBα, the natural inhibitor of NFκB, revealed reduced expression of

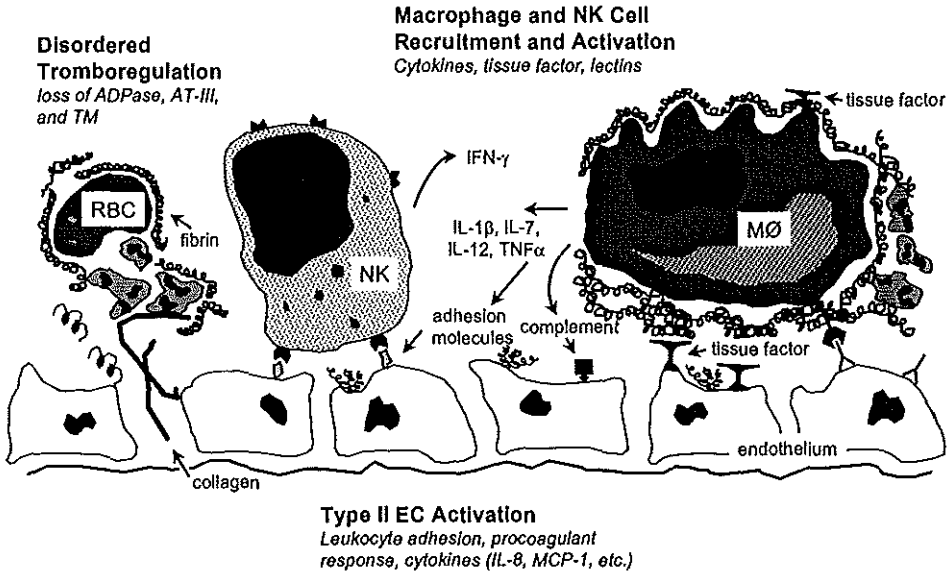

Figure 4. *Model of type II endothelial cell activation and delayed xenograft rejection.*

Thrombosis occurs both on events that are part of type I endothelial cell activation (loss of ADPase, anti-thrombin III (AT-III) and thrombomodulin (TM)) and on expression of tissue factor, which is expressed on both graft EC and graft-activated monocytes (MØ). Multiple inflammatory mediators, produced by activated EC and platelets, recruit and activate inflammatory cells. Preformed natural antibodies are able to bind MØ. NK-cells bind specific carbohydrates presented by EC [156,157]. Activated monocytes and NK-cells produce cytokines, which stimulate activation of EC and infiltrating cells [158]. Complement derived from MØ might increase the injury [146]. (adapted from Bach et al. [20])

those molecules but increased apoptosis [154]. Introduction of p65RHD resulted in suppression of the same genes without apoptosis [165]. Another strategy concerns the introduction of anti-apoptotic genes [166,167]. Anti-coagulation therapy results in prolonged discordant graft survival and at rejection reduced platelet aggregation was seen [134-136]. In addition, expression of human thrombomodulin cofactor activity on porcine endothelial cells reduced thrombin-induced platelet activation [155].

NK-cell activation is inhibited by MHC class I molecules via killer inhibitory receptors. Due to molecular incompatibilities this function could be disturbed [168]. Introduction of recipient MHC I substantially blocked the cytotoxic effects of NK-clones [169]. Overexpression of IκBα inhibited NK-cell mediated endothelial cell activation [170]. However, 'just' blockade of xenoantibody production and anti-NK-cell and nitric oxide treatment still led to xenograft rejection, although postponed [171].

Other approaches include the induction of "accommodation", i.e. the situation in which the grafted organ does not reject, although specific antibodies and/or complement are present, or "tolerance", i.e. the specific immunosuppressive free situation in which the immune system does not reject the grafted organ.

Accommodation, first described by Bach et al. [58], describes a phenomenon in which a transplanted organ is allowed to 'heal in' after grafting. Retransplantation experiments demonstrated that this process is seen at the level of the xenografted organ by expression of 'protective genes' [172]. *In vitro*, Dalmasso et al. found that endothelial resistance could be achieved through the use of human IgM natural antibodies in the absence of complement [173]. Mechanisms of accomodation might include a role of low levels of anti-donor IgG antibodies or the upregulation of protective genes [174,175].

One approach to tolerance induction is the achievement of mixed chimerism. Donor bone marrow has been grafted into the recipient to induce an immune cell repertoire that is tolerant to the grafted organ from the same donor [176,177]. Discordant skin graft tolerance and long-term porcine to monkey bone marrow engraftment indicates that xenotolerance is possibly a promising approach [176,178].

Of course the latter strategies will not focus on DXR alone, but will include the T-cell-mediated response in xenotransplantation.

T-cell response in xenografting

The cellular response was never thought to be important because of inadequacy of T-cell receptors to recognise non-self MHC and the disparity of molecular interactions involved, such as accessory molecule and lymphokine interactions. Different *in vitro* assays demonstrated not only diminished frequencies of responder cells [179,180] but also reduced cytokine interactions [181-183]. More recent studies demonstrate that several T-cell interactions are intact in the human anti-pig response [184,185]. Murray et al. reported that the human anti-pig cellular response is mediated both by direct and indirect pathways of recognition [186]. Recent studies have reaffirmed these findings [187,188].

How the cellular rejection arises *in vivo* is still unknown. It appears that the responses seen *in vitro* require immunosuppressive regimens at least as potent as those used in allografting [189-191]. To study the cell-mediated xenograft rejection *in vivo*, some experimental models have been developed [192,193]. After limb xenotransplantation, vigorous immunosuppression seems to be required [194].

Complement also plays a pivotal role in discordant xenografting. Even in animals transgenic for complement regulatory proteins inactivated complement residues might enhance cellular rejection. Membrane-bound breakdown products of C3 enhanced

immunogenicity by 1.000- to 10.000-fold [195,196] and, therefore, possible enhance T-cell rejection.

Even chronic rejection of xenografts is under investigation. Several studies support the view that xenografts will be subjected to powerful chronic rejection [197-200].

Microbiological and political considerations

Until recently, it was thought that the risk of infection after xenotransplantation was negligible. The pig, the most likely donor in clinical xenografting, has lived alongside humans for so long that by now we would have picked up any of their microbes capable of infecting us. However, natural immunological barriers like skin and mucosa are not present in transplantation. Secondly, the immunosuppression needed to prevent graft rejection will help a virus to propagate and adapt to its new host. Thirdly, human genes bred into transgenic donor pigs could promote pre-adaptation of animal viruses and cause human infection [201]. CD55 and CD46, both complement regulatory proteins, act as receptors for viruses capable of causing myocarditis or measles [202].

Pig viruses may not be recognised if they do not cause disease in pigs, such as the pig calcivirus only discovered two years ago [203]. Furthermore, pigs contain 'endogenous' retrovirus genomes that are inherited in the DNA of normal pig chromosomes [204]. Infection of human cells by pig endogenous retroviruses has already been reported [19]. Nevertheless, humans have not been infected by the already performed pig islets of Langerhans transplantations and extracorporeal pig organ perfusions [18,205,206]. This could be due to the short exposure to the used pig tissue.

Not only because of the potential risk of infection for the xenografted patient but also for the public, many governments, including the Dutch, have issued guidelines aimed at tightening regulations for xenotransplantation. Committees are set up to review the development in xenotransplantation and authorise clinical trials. Preferably, primates will not be used as donors, but so far no government has banned them. The guidelines further call for long-term surveillance of xenograft recipients and the welfare of the porcine donors. In the Netherlands an appointed Committee advised the government not to endorse clinical xenotransplantation as knowledge and immunosuppression of the designated rejection processes are still poor.

Due to the upheaval that retroviruses might infect human cells [19,207], clinical experiments have been postponed. Recently in Berlin, however, extracorporeal pig organ perfusions have been resumed.

The liver

The liver is the largest gland of the body and can be regarded as the central organ in metabolism. It continuously supplies the organism with energy substrates. During intestinal absorption excess glucose is stored as glycogen in the liver. To maintain the energy supply of glucose-dependent organs, glucose is formed by glycogenolysis and gluconeogenesis in the post-absorption phase. The liver is also the major site in amino acid degradation and transformation. Moreover, the liver has biosynthetic functions and plays a key role in regulating plasma hormone levels. For the synthesis and secretion of plasma proteins, like clotting factors and complement components, amino acids are used.

In detoxification of endogenous and exogenous products, the liver is also the central organ. Besides the urea cycle and glutamine formation, plasma proteins are degraded and endogenous degradation products such as bilirubin are solubilised for excretion.

Parenchymal and non-parenchymal cells are involved in the diverse functions of the liver. Neither of them represents homogeneous cell populations. Parenchymal cells (hepatocytes) account for 60 percent of the total cell population [208]. Differences in hormone and substrate concentrations, innervation, biomatrix and microenvironment may be responsible for the expression of metabolic heterogeneity. The large population of non-parenchymal cells is represented by at least four different cell types being endothelial cells, Kupffer cells (macrophages), stellate cells (Ito-cells, fat storing cells, perisinusoidal cells) and pit cells (NK-cells). Kupffer cells, tissue fixed macrophages, make up 10 percent of these cells and have a life span of about 14 months [209]. Their main function is the clearance of pathogens via Fc- and C3b-receptors [210,211]. Synthetic properties of the liver include the production of complement factors, cytokines, acute phase proteins and oxygen radicals. Apart from filtration capabilities, sinusoidal endothelial cells are able to degrade soluble immune complexes. In the rat and mouse the sympatic innervation is restricted to the periportal area, whereas in the human, dog, cat, rabbit and guinea pig all hepatocytes are innervated.

Functional and histological structures

Liver function is defined as the capacity to regulate the concentration of substances in the hepatic venules and bile. To accomplish this function several mechanisms are present: 1. transport of substances, such as uptake, intracellular movement and secretion, 2. biotransformation, 3. synthesis and 4. storage.

At the hilus, poorly oxygenated blood enters via the portal vein. It supplies approximately 80% of the blood. The hepatic artery provides the well-oxygenated

remainder of the total supply to the liver. Efferent bile ducts and lymphatics exit the liver at the hilus. Venous drainage, the inferior vena cava, runs independent of the above structures. Within the liver, the portal vein, hepatic artery and bile duct course in parallel. After repeated branching the vessels supply blood to the sinusoids, which are the vessels involved in transvascular exchange. The sinusoids drain in the terminal veins of the hepatic vein.

The structural and functional units of the liver can be described from different concepts (see Figure 5). Historically, Kiernan proposed the classic lobule [212]. Rappaport described a second model, the liver acinus [213]. Both concepts do not describe the minimal amount of tissue that is required to perform all functions. The 3-dimensional sickle zone model was described by Matsumoto and Kawakami [214] and seems to incorporate all concepts lacking in the other two.

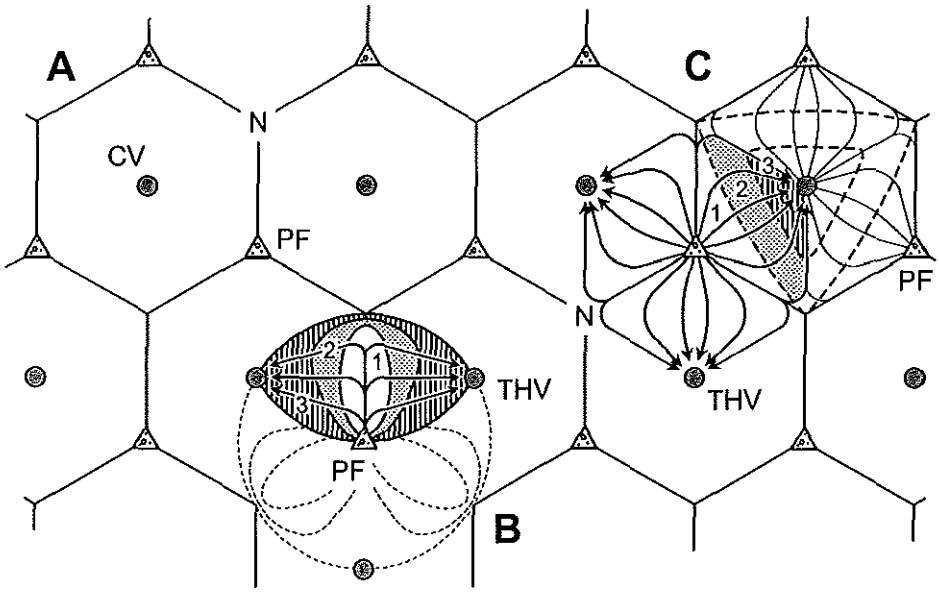

Figure 5. *Models of structural and functional units of the liver.*
The classical lobule (A) embodies a central vein (CV) and portal field (PF), which incorporates a portal vein, hepatic arteriole and bile ductile. The liver acinus (B) consists of PF and terminal hepatic vein (THV) and can be divided in metabolic zones (1, 2 and 3) that differ in oxygenation grade. The sickle zone model (C) describes cone-shaped subunits with haemodynamic equipotential lines/zones (1, 2, and 3) in a primary lobule. (adapted from Katz and Jungermann [215])

Biosynthesis of complement

The liver is the main source of complement. This notion was first published by Ehrlich and Morgenroth [216] and further evidenced by Olsen [217]. Nowadays, it is accepted that indeed the majority of complement components are synthesised by the liver [218]. It is well known that hepatocytes of transplanted xenografts retain their metabolic specificity [9,219]. As the complement components are mainly synthesised by the liver, the new supply of donor-specific complement might be a survival advantage for the transplanted liver. This thought was supported by Valdivia et al. who reported that the concordant xenograft recipient had two complement systems of which the dominant one was donor [220]. In discordant liver grafting, however, an extra disadvantage might be encountered due to the molecular incompatibility of the complement systems and their regulators. Extrahepatic complement synthesis might be able to reject xenografted organs [147], whereas hepatic complement might cause rejection of host organs.

Aims of the thesis

Liver transplantation is indicated and an accepted form of therapy for patients suffering severe liver insufficiency. Over the last decades the clinical outcome has improved due to new immunosuppressives, technical progress and insight in liver graft rejection. Therefore, the number of patients being considered for transplantation has increased. The availability of donor livers, however, is limited, which leads to possible lethal situations for patients with acute liver failure. Despite numerous efforts to structurally solve the donor shortage, the discrepancy between supply and demand is not declining but unfortunately increasing. Xenotransplantation, grafting of organs between species, may offer a possible solution. At the beginning of the transplantation era, animal organs were used for grafting in man with variable success [7,221,222]. Because of the limited availability of donor organs, renewed interest in xenotransplantation arose.

Xenotransplantation between widely disparate species is hindered by hyperacute rejection and the following delayed xenograft rejection. Preformed xenoreactive natural antibodies play a main role in both processes. Clinically, it has been noticed that livers, but not other organs, can be transplanted across an ABO barrier and against T-cell cross-matches [223-226]. In experimental studies, liver grafts were also relatively insensitive to antibody-mediated rejection [227,228]. In concordant liver grafting, survival times indicated that this phenomenon may exist in xenografting [229]. This may be of importance for liver xenotransplantation with respect to the antibody barrier.

Transplantation of organs across species entails a question of compatibility not seen in allotransplantation: the ability of an organ to perform its physiological function in the new host environment. Little attention was focussed on this subject [230]. Evolutionary considerations, though, indicate that physiological complications arise after xenogeneic transplantation [230,231]. In 1990, early indications of physiological incompatibility were already found to exist in the hyperacute rejection process. After discordant grafting, massive complement activation is seen, caused by disordered regulation of the complement system [30]. Due to its complex metabolic function, liver xenografting might encounter extra difficulties with respect to physiological compatibility.

Therefore, the aim of the present thesis consists of two subjects:

A. Are liver grafts less susceptible to antibody-mediated rejection in a xenogeneic setting?
B. Are xenogeneic liver grafts physiologically compatible with their new environment in non-concordant hosts?

As the pig seems to be the most likely candidate for clinical xenotransplantation, the emphasis of this thesis was on discordant xenografting.

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Surgery

Technical aspects of experimental orthotopic liver transplantation in the rat

Introduction

In the 1950's, the first experiments were started to develop liver transplantation. It was not until 1967 that the first patient survived 1 year after a successful liver transplantation [1,2]. A few years later, the first rodent liver transplantation model was developed [3]. Kamada et al. considerably shortened the anhepatic phase in the recipient using a cuff-technique enabling an excellent long-term survival rate [4]. The cuff-technique, however, requires a high level of microsurgical skill and extensive training [5].

Orthotopic liver transplantation (OLT) was a technique new to our research group. In contrast to heterotopic heart transplantation as described by Ono and Lindsey [6], life-supporting and metabolic functions of liver grafts complicate successful transplantation. Therefore, to optimise and become skilled at the technique, several modifications of the OLT method were attempted.

Surgical attempts and modifications

Hepatic artery

The liver holds a peculiar position with regard to its blood supply. It is the only organ that obtains venous blood, via the portal vein (PV). In the rat, the hepatic artery provides only 10% of the total liver blood supply. In OLT, arterial reconstruction has been an object of research for many years. Several authors have found histological abnormalities and even reduction in immunological response and diminished survival were noticed after OLT without arterial blood supply [7-9].

In the rat model, hepatic artery flow has been re-established by using an end-to-side aorta-aorta anastomosis [10], a hepatic artery-celiac artery cuff method [11], an aorta-right renal artery cuff technique [12], and a celiac artery-right renal artery sleeve anastomosis [13]. Due to its time-consuming character and complications, like thrombosis, arterial reconstruction was abandoned. Moreover, biliary complications and differences in survival and histological appearance appear to be technique-related rather than the result of ischaemia [14]. Especially in studies that include short-term graft function, like xenotransplantation, rearterialisation is not necessary [15].

Bile duct

Lee et al. used a technique were the bile duct was implanted in the duodenum of the rat [3]. Elevation in serum bilirubin was frequently seen [3]. Kamada et al. developed a two-sided fitting splint [4]. However, certain complications also occurred in this model [16]. The one-splint technique described by Dippe et al. [10] was adopted without serious problems.

Cuff technique

At first, OLT was performed using running sutures on the suprahepatic and infrahepatic vena cava (SVC, IVC, respectively) and PV. The time between the moment of clamping the PV and reperfusion of the liver graft, the anhepatic phase, is rather critical. Periods longer than 26 minutes clearly diminished recipient survival [16]. Using running sutures for the PV and SVC, this time is amply surpassed. To overcome this problem a portosystemic shunt was used [3]. However, in our model thrombosis in the shunt hindered the surgical procedures seriously. Therefore, the cuff technique for the PV and IVC anastomosis, proposed by Kamada et al. [4], and the SVC, proposed by Miyata et al. [17] were used. The advantage of this technique is that the anastomosis can be carried out within a few minutes without the introduction of artificial materials. A disadvantage of this elaborate technique is that relatively large donor vein stumps are required, because the vein is pulled through and slipped inside out over the cuff (see Figure 1A-I). The cuff itself has to be rather stiff to perform the technique and must have a high inner/outer diameter ratio to exclude venous stricture. As the vein is pulled through the cuff, ligated side-branches have to be trimmed off to prevent venous obstruction within the cuff caused by these ligations.

As the SVC is very short between the liver and diaphragm, a thoracic part is used. However, this part of the vena cava is almost half the diameter of the SVC directly above the liver. In our model, OLT performed as described by Miyata et al. and Settaf et al. [17,18], demonstrated severely impaired haemodynamics. The SVC cuff was, therefore, abandoned.

The anhepatic phase with a running suture for the SVC and cuff for the PV varied between 19 and 24 minutes.

Transplantation technique

Donor operation

Female donor hamsters were used, because their body weight and, consequently, their liver size is larger than of male hamsters. In discordant grafting, donor guinea pigs (GP) of only 2 to 4 weeks old were operated on, because of their relatively large livers. Brown Norway (BN) rats were used as liver graft recipients. Lewis rats were found to be unsuitable in our hands, because cross-clamping the diaphragm resulted in cessation of respiration.

In this report, the technique of rodent liver transplantation is described using the hamster to rat model as example, which is portrayed in Figure 2.

Donor hamsters were pre-treated with a 0.1 mg/kg atropine im. bolus injection to prevent extensive saliva production due to ether anaesthesia. The abdomen, including

the lateral sides, was shaved and disinfected with alcohol. After an abdominal transversal incision, the skin and the xiphoid were retracted to expose the liver. The intestines were externalised toward the left, exposing the portal vein. The liver was dissected free from the hepatophrenic, hepatogastric and SVC ligaments and all post-hepatic veins were coagulated. The left phrenic vein was ligated using 6-0 silk (B. Braun, Melsungen AG, Melsungen, Germany) and cut (see Figure 2A). By brief displacement of the liver to the left side, the retroperitoneal ligaments were exposed and cut. To dissect the IVC, right adrenal vein, left and right renal veins and lumbar veins were ligated, using 8-0 nylon (B. Braun-SSC AG, Neuhausen am Rheinfall, Switzerland) or 6-0 silk sutures (vein size dependent), and cut. The PV was mobilised between the portal bifurcation and the hilus, ligating the pyloric, splenic, and inferior mesenteric veins with 8-0 nylon. To avoid bleeding, great care was taken in dissecting the portal vein. To prevent future reduction of the vein lumen inside a cuff, all connective tissue was removed from both PV and IVC. Subsequently, a transversal incision in the bile duct was made. A handmade 8-mm long Teflon splint (Medica B.V., 's Hertogenbosch, The Netherlands) with a diameter of 0.63 mm was introduced and secured by a circumferential 6-0 suture. A residual 5 cm suture material was kept, allowing traction in the recipient operation. The hepatic artery was coagulated. After injection of 75 IU heparin iv., the IVC and PV, with its venous branches, were cut. Over a period of 2 minutes, the liver was flushed *in situ* with 6 ml of ice-cold University of Wisconsin solution (UW) via the PV (see Figure 2B). The SVC was cut as close to the diaphragm as possible. The liver was immersed upside-down in an ice-cold saline bath displaying the PV and IVC. The whole donor procedure took about 50 minutes.

When the GP is used as donor, modifications are as follows. For anaesthesia, 0.6-1.3 ml 48% w/v urethane (Sigma, St. Louis, USA) was used. Extra care was taken with the dissection of veins. The vascular wall is very thin and the surrounding tissue is tougher then in hamsters, increasing the risk of vascular bleeding. Together with the heparin infusion, 5 mg phentolamine mesilate (Ciba-Geigy, Basle, Switzerland) was injected intravenously to accomplish vascular dilation in the liver. Instead of 6 ml UW, 8 ml were flushed through the liver.

***Ex vivo* graft preparation**

A clamp was positioned to hold the cuffs and provided a stable platform to perform the cuff technique (see Figure 2D). The Teflon cuffs were handmade from tubes (Medica B.V., 's Hertogenbosch, The Netherlands) and had an outside diameter of 1.79 mm and 2.1 mm for the PV and IVC, respectively. The cuffs were modelled and the surface was made rough with fine sandpaper. After positioning of the cuff over the saline bath, the vein was pulled through the cuff using forceps (see Figure 1A, B, C). Two forceps were used to spread the lumen of the vein (see Figure 1D). The vein was everted over the cuff

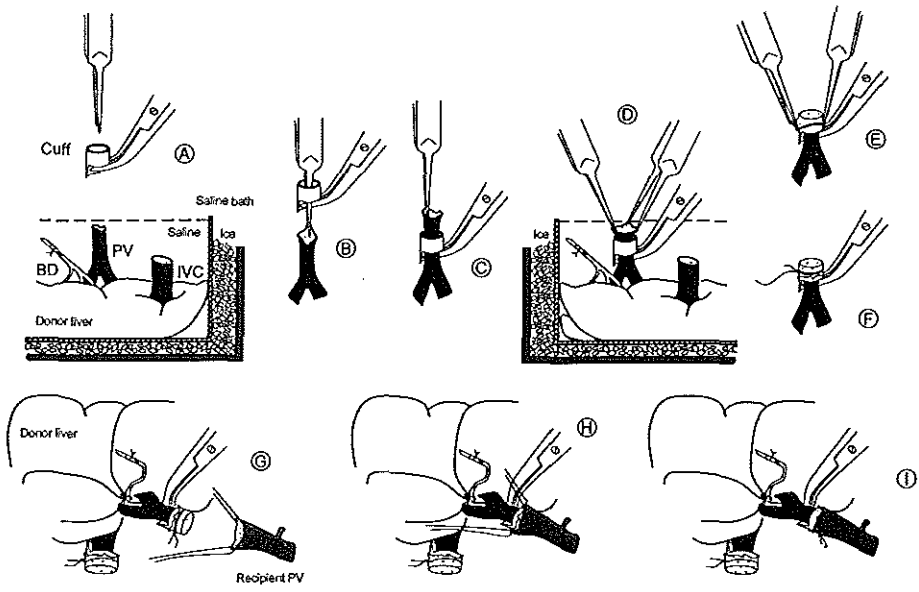

Figure 1. *The cuff technique during cold ischaemia and in transplantation.*
The cuff technique is used for both portal vein (PV) and infrahepatic vena cava (IVC). The bile duct (BD) is already cannulated with a splint during the donor procedure. (A) The liver graft is placed in an iced saline bath. A clamp, "third hand", holds the cuff in position. (B) Forceps are passed through the cuff to grasp the vein. (C) Then the vein is pulled through the cuff and (D) another forceps helps to spread the lumen. (E) The vein is slipped inside out over the cuff and (F) secured with a circumferential suture. (G) *In vivo*, the cuff is immobilised by a clamp. Two traction sutures are positioned in the vein (recipient PV). (H) The cuff is introduced in the vein and (I) secured with a circumferential suture. (adapted from Kamada et al. [4])

and a circumferential 8-0 nylon suture with a double surgical knot and a two simple knots was used to secure the vein (see Figure 1E, F and Figure 2C). This procedure was performed for both the PV and IVC. Finally, a clamp was placed on the IVC and the bile duct leading to the gallbladder was coagulated. The total cuff preparation took about 15 minutes.

Cuffs used for GP donors were 2.3 mm and 2.1 mm for the PV and IVC, respectively. The bile duct splint had a diameter of 1.1 mm.

Recipient operation

The BN rat was anaesthetised with ether. After shaving and disinfecting the abdomen, the rat was placed on a heating plate (max. 38°C). An abdominal midline incision was made from the pubis to xiphoid. Retraction of the abdomen and xiphoid exposed the liver. It was dissected free from all above-mentioned ligaments and all post-hepatic veins were coagulated. The right adrenal vein was coagulated and the IVC was

mobilised between the liver and the right renal vein. The portal vein was freed from surrounding tissue between the hilus and gastric vein. The hepatic artery was coagulated. Then the bile duct, superior mesenteric artery, IVC, PV and SVC were clamped in that order. The artery clamp was placed to reduce bowel and portal vein congestion. The IVC was clamped as distally as possible, but above the right renal vein. After positioning the portal clamp over the gastric and portal vein, the anhepatic phase started. During the anhepatic phase, ether anaesthesia was almost superfluous. By displacing the liver distally, a Satinsky clamp was placed over the diaphragm to clamp the SVC and phrenic veins. All three veins, IVC, PV and SVC, and the bile duct were cut as close to the liver as possible. The liver was rapidly removed, small haemorrhages were coagulated and the cavity and veins cleaned with saline (see Figure 2E). The liver graft was placed orthotopically and cooled with saline gauze. The recipient was turned 180° and two 8-0 sutures were placed on the left and right side of the SVC. The SVC was anastomosed using an 8-0 running suture, leaving the left phrenic vein intact. Care was be taken to prevent air embolisms. Taking advantage of small residual liver tissue on the recipient SVC and the natural very low blood pressure in the SVC, relatively large stitches could be placed. On the recipient PV, two traction sutures were applied (see Figure 1G). The PV cuff was introduced in the recipient PV using a clamp and irrigated to avoid air bubbles (see Figure 1H). After positioning the cuff two circumferential sutures, 6-0 and 8-0, were applied (see Figure 1I and Figure 2F). The clamps of the PV, SVC, and superior mesenteric artery were removed allowing reperfusion. Reperfusion ended the anhepatic phase. The traction sutures were removed. The same technique as used for the PV was applied for the IVC. After re-establishing the vena caval flow, in most cases the liver was already producing bile (see Figure 2G). Now, traction was put on the donor bile duct. With two forceps, the recipient bile duct was slipped over the splint and secured with 6-0 suture. To prevent slipping of the bile duct from the splint, both sutures were tied to each other. The abdomen was closed using a continuous 2-0 silk suture (B. Braun, Melsungen AG, Melsungen, Germany). The animal was kept warm until full recovery and received 5 ml of warm glucose 5% with 0.1 ml of the antibiotic procaine, penicillin G and dihydrostreptomycin (P.P.S) subcutaneously (Apharmo, Arnhem, The Netherlands). The recipient transplantation procedure lasted about 60 minutes of which 20 to 24 minutes were anhepatic.

This technique resulted in indefinite survival of liver grafts in the syngeneic BN to BN and allogeneic PVG to BN rat combination.

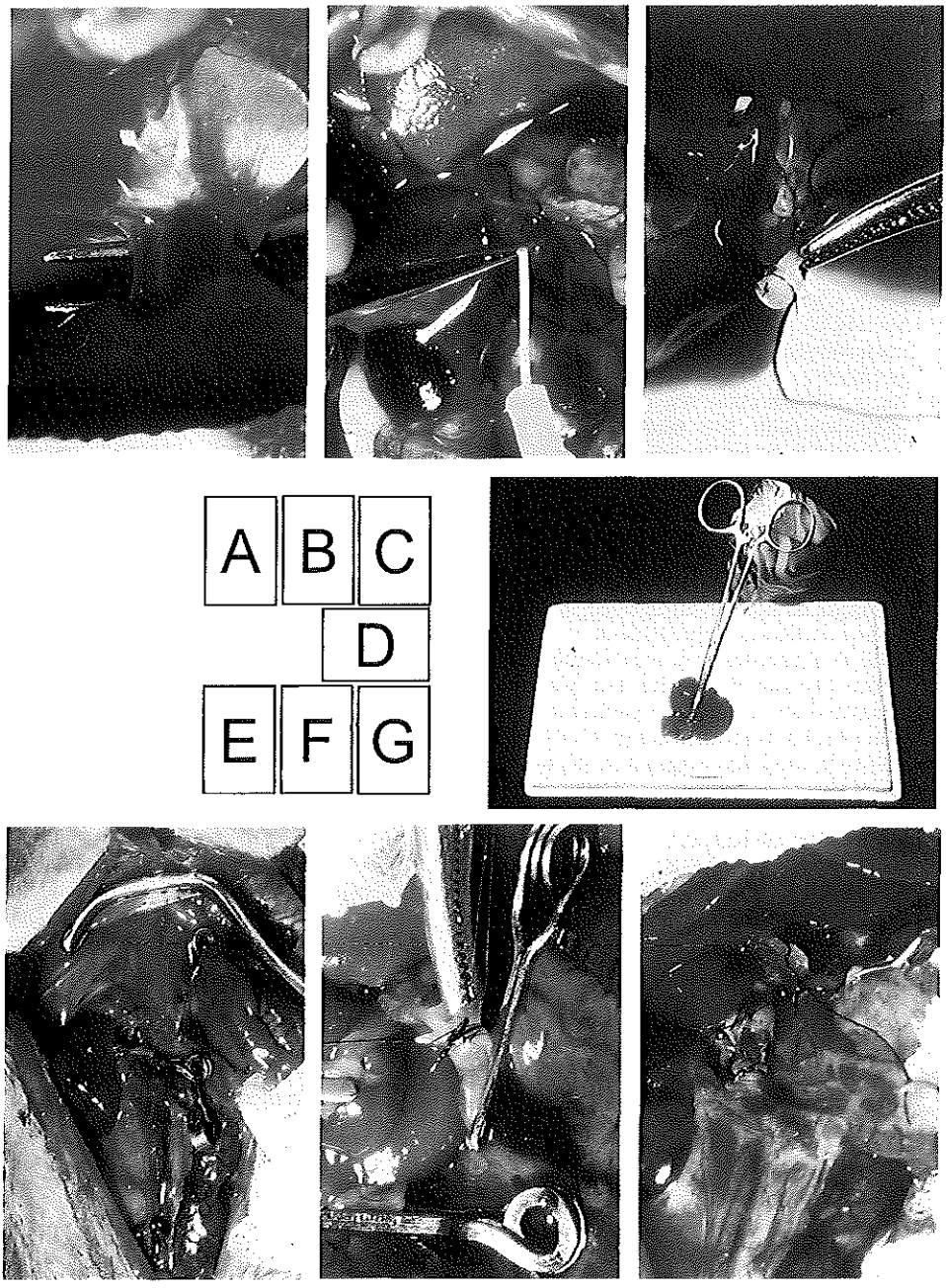

Figure 2. *Photos of the orthotopic transplantation technique in the rat.*
Dissection of the suprahepatic vena cava (A), perfusion of the donor liver with preservation fluid (B), portal cuff (C), *ex vivo* graft preparation (D), abdomen of the rat after removal of the host liver (E), *in vivo* anastomosis of the portal vein (F) and reperfused grafted liver with anastomosed portal vein and infrahepatic vena cava (G).

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Part A

Susceptibility of the liver to antibody-mediated rejection in xenotransplantation

CHAPTER One

Prolonged graft survival after orthotopic liver transplantation in the hamster to rat model

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Abstract

Transplantation as a therapy in endstage liver disease is hampered by donor shortage. Animal organ donors may offer a solution, but severe immunological processes ensue upon transplantation. The guinea pig (GP) to rat heart transplantation model is often used to study these processes. Clinically, the liver is less susceptible to antibody-mediated rejection. The existence of this phenomenon in xenografting might benefit future clinical practise. However, the technical feasibility of GP to rat liver transplantation is largely unknown. In the hamster to rat model, several groups have described long-term liver graft survival. To investigate the feasibility in our model and the suspected immuno-privileged position of the liver, hamster livers and hearts were grafted.

Eight groups were studied. In the untreated group, mean survival was 3.9 and 6.8 days for heart and liver grafts, respectively. Cyclosporine A (CsA) treatment (25 mg/kg) did not prolong survival. An increase in the number of recipients that died with functioning grafts was noticed after TBI treatment, especially in the liver transplantation groups. Nevertheless, total body irradiation (TBI) of 5 Gy significantly prolonged heart graft (>13.7 days). The combination of CsA and TBI revealed similar (early) deaths in heart and liver graft recipients (heart: >20.8 days, liver: >7.3 days). For liver transplantation, an extra FK506-treated group was added. In this group, recipients demonstrated prolonged to long-term graft survival (mean survival 31.4 days), which is comparable to results found by others. Graft rejection correlated with histological changes and levels of liver enzymes ALAT, ASAT, AF, γGT, tBIL, and AP.

In conclusion, although surgical trauma and immunosuppressive side-effects have a larger impact in liver xenografting than in heart grafting, hamster to rat liver xenotransplantation is technically feasible with similar results as found by others.

Introduction

Liver transplantation is an accepted therapy for patients with endstage liver disease. Due to the increasing demand, there are not sufficient organs for every potential transplant patient. Grafting of livers across a species barrier may be the ultimate solution to this problem. However, xenotransplantation is hampered by strong immune responses. Discordant grafting, hindered by specific anti-donor antibodies and complement disparity, results in hyperacute rejection. After concordant grafting, induced antibodies and cellular rejection processes are initiated leading to accelerated acute rejection. Moreover, there is a possibility of non-functioning in a xenogeneic environment, due to physiological differences and molecular disparities.

The guinea pig (GP) to rat transplantation model is often used as a model for discordant grafting. The rat recipient has preformed xenoreactive natural antibodies and complement activation is seen after heart grafting resulting in rejection within minutes [1,2].

Clinically, it has been observed that liver, in contrast to other organs, can be transplanted across an ABO barrier and against T-cell cross-matches [3]. This suggests that the liver is relatively insensitive to antibody-mediated rejection. If an immuno-privileged position exists for liver xenografting, this might be of benefit for future clinical practise. The analysis of the technical feasibility of liver xenotransplantation, i.e. transplantation techniques and rejection parameters, has to be done by reproducing and comparing the results found by others. However, publications on discordant liver grafting in the GP to rat model and information on model specific characteristics are rare. In the concordant grafting, publications on liver transplantation are more plentiful. In addition, it has been found that concordant liver grafts may also be less susceptible to antibody-mediated rejection [4].

Therefore, the aim of the present study was to investigate the technical feasibility of liver xenografting and the possible immuno-privileged position of liver grafts to antibody-mediated rejection.

Materials and methods

Animals

For the transplantation procedure, female Syrian hamsters were used as donors. The weight of the animals was 130 grams on average. Male inbred Brown Norway rats were used as recipients of liver and Lewis rats as recipients of heart grafts. Animals of both strains weighed between 250 and 300 grams. All animals were obtained from Harlan C.P.B. (Austerlitz, The Netherlands). They were kept under controlled laboratory conditions and received food and tap water ad libitum. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). The "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands, approved the specific protocol.

Procedure liver transplantation

Orthotopic liver transplantation (OLT) was performed according to Kamada et al. with some minor modifications [5,6]. Atropine was used to pre-treat the donor. The diameters of the Teflon cuffs used for the anastomoses were 2.1 mm and 1.79 mm for the infrahepatic vena cava and portal vein, respectively. The bile duct splint had a diameter of 0.63 mm. The donor operation took about 45 minutes, the cuff preparation 15 minutes, and the recipient procedure about 60 minutes. The anhepatic phase did not exceed 25 minutes. Photos of crucial moments in the procedure are portrayed in the chapter denoted as "surgery". Recipient death was taken as endpoint of rejection.

Procedure heart transplantation

Heterotopic intraabdominal heart transplantation (HTx) was performed as described by Ono and Lindsey [7]. The donor operation lasted about 10 minutes and the recipient procedure about 45 minutes. Cessation of heart beating, assessed by palpation, was taken as endpoint of graft survival.

Liver enzyme monitoring

Liver enzymes were monitored just before and after transplantation. As parameters of cellular damage, serum alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined. Alkaline phosphatase (AP), γ-glutamyl transpeptidase (γGT) were used as parameters indicating bile duct obstruction. Total bilirubin (tBIL) and cholinesterase (CHE) were measured as parameters for liver function.

Histology

After rejection, necropsy was done. The heart graft or upper liver lobe was removed and processed for histology. Formalin fixed tissue was paraffin embedded, cut and stained with haematoxylin-eosine for examination of morphological changes by conventional light microscopy.

Experimental design

Heart and liver transplantations were carried out in four and five groups, respectively. Recipients in group 1 and 5 (n=7 for heart and n=9 for liver, respectively) received no treatment. Intramuscular administration of 25 mg/kg cyclosporine A (CsA), on days -2, -1, 0, and thereafter 3 times a week, was added to achieve T-cell inhibition (n=8 for heart, group 2; n=7 for liver, group 6). In groups 3 and 7 (n=7 for heart; n=6 for liver, respectively), recipients received total body irradiation (TBI) of 5 Gy on day -2 using a ^{137}Cs gamma source. Rats in groups 4 and 8 (n=8 for heart, n=9 for liver, respectively) received the combination of CsA and TBI.

In the ninth group 2 mg/kg tacrolimus (FK506) was administered daily for 2 weeks. Thereafter, liver recipients received 1 mg/kg FK506 until day 28, followed by 0.5 mg/kg on alternating days.

Survival time (abdominal palpation or recipient death) was scored in days. Heart and liver grafts were examined for morphological changes.

Statistics

Statistical evaluation was performed using logarithmic transformation values of the survival data. This was done to reduce the influence of outlying values. "One-way" analysis of variance (ANOVA) was performed on these data. If the ANOVA was significant on a 5% level, post-hoc tests were carried out. Depending on the homogeneity of variances, tested by the Levene test, Games-Howell test or Duncan's multiple comparison test were carried out for possible differences among the means. These tests were corrected for unequal group sizes.

The T-test was used to compare the logarithmic transformation values from survival data of heart and liver grafts within one treatment.

Probability values lower than 0.05 were considered statistically significant. Survival of transplants with clear evidence of no rejection (liver enzymes, histology) was discarded from statistical evaluation. All computing was done using the statistical software package SPSS for Windows, release 7.5.2.

Results

Graft survival

All liver grafts showed a homogeneous reperfusion after releasing the clamps. Around 55% of the liver graft recipients died without the occurrence of rejection.

Graft survival times are shown in Table 1. A highly significant difference was found between the group means in the heart transplantation experiments ($p=0.000$, $s_{res}=0.276$). Exclusion of the non-rejected recipient survival times resulted in a significant prolongation of survival times for the combination treatment, compared to the untreated and CsA-treated groups $p=0.000$ for both). The TBI-group was excluded from comparison because no statistical variance could be calculated. As seen by minimal survival times in the TBI and combination treatment groups, inclusion of them in the statistical analysis demonstrated significantly prolonged survival times for groups 3 and 4, compared to groups 1 and 2 ($p=0.000$ for all).

Table 1. *Survival times following concordant heart and liver transplantation.*[c]

Group	Surgical procedure	Treatment	Survival[a] (days)	Mean survival
1	HTx	Untreated	3, 4, 4, 4, 4, 4, 4	3.9
2	HTx	CsA	4, 4, 4, 4, 5, 5, 5, 5	4.5
3	HTx	TBI	11, (14), (14), (14), (14), (14), (15)	>13.7[b]
4	HTx	CsA + TBI	(10), 11, (13), (19), (23), (24), (24), 42	>20.8[b]
5	OLT	Untreated	(2), (2), (2), (2), 6, 6, 7, 7, 8	6.8
6	OLT	CsA	(1), 6, 6, 7, 8, 8, 8	7.2
7	OLT	TBI	(1½), (1½) hours (1), (1), (2), (2)	-
8	OLT	CsA + TBI	(1), (1), (1), (3), (3), (4), 5, (6), (11)	>7.3[b]
9	OLT	FK506	(3), (4), 9, 10, 28, 30, >80	31.4

[a]Animals that died with functioning grafts are put between parenthesis.

[b]Mean survival was calculated using all survival times with a minimum of 3 days for heart grafts and 5 days for liver grafts.

[c]Grafts were transplanted in the hamster to BN transplantation model. Cyclosporine A (CsA, 25 mg/kg) (groups 2 and 6) was administered on days -2, -1, 0, and thereafter 3 times a week. Total body irradiation (TBI, 5 Gy) (groups 3 and 7) was performed on day -2. Groups 4 and 8 received the combination treatment. Starting from day 0, daily tacrolimus (FK506, 2 mg/kg; after day 14: 1 mg/kg) and after day 28 3 times a week (0.5 mg/kg) was given to group 9. Significant differences concerning heart graft survival were found between the CsA/TBI-treated group and the untreated and CsA-treated groups. In the liver grafted groups, significantly prolonged survival was seen after FK506-treatment compared to no or CsA-treatment.

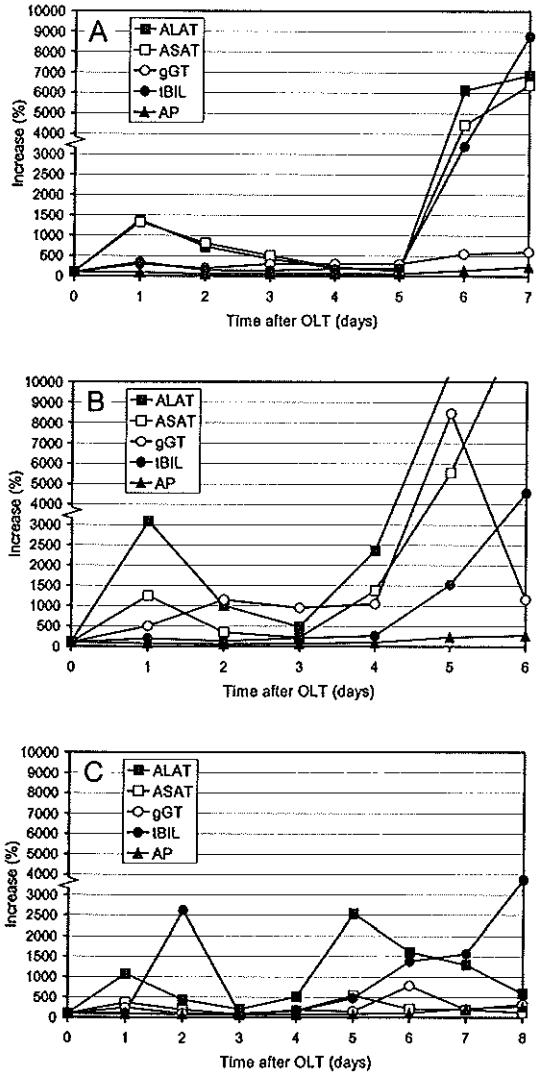

Figure 1. *Relative enzyme and protein levels in liver graft recipients.*
Representative animals were chosen from the untreated (A), the CsA-treated (B), and the combination (CsA/TBI) treatment group (C).

A significant difference was found between group means after liver grafting ($p=0.003$, $s_{res}=0.511$). FK506-treatment significantly prolonged survival times when compared to untreated and CsA-treated recipients ($p<0.005$ for both). No comparison was made for TBI-and TBI/CsA-treated groups, because of the absence of statistical relevance. When the minimal survival times of the combination treatment were included, no extra significant differences were found. It was noted that schedules, which included TBI-treatment, resulted in liver graft recipient death within 1 day after transplantation in most cases.

Liver enzyme monitoring

Liver enzymes of representative animals from groups 5, 6 and 8 are shown in Figure 1. Liver enzymes, as measured by a 50-fold rise in ALAT and ASAT, indicated rejection in the recipients from the untreated and CsA-treated groups. Low bilirubin clearance indicated severely impaired liver function upon rejection. No synchronous elevation of tBIL and AP was noted, indicating that no bile duct obstruction could be noted before rejection. In the recipient from group 8, fluctuation of several parameters could be noted but returned to normal after 1 to 2 days. This indicated that no acute rejection was ongoing.

Normal CHE-activity in hamsters is about 2 to 3 times higher than in rats. After grafting, CHE-activity remained unchanged.

Histology

Specimens of the transplanted organs for histological examination were obtained after sacrificing or death of the animals. Control heart grafts showed extravasation, oedema and vascular destruction, primarily indicating an antibody-mediated rejection. No other histological changes were seen after CsA-treatment, compared to the untreated group. Heart grafts slides demonstrated slight monocyte infiltration after TBI-treatment. In the combination therapy group histological examination revealed a mixture of findings ranging from no morphological changes to severe infiltration by polymorphonuclear granulocytes and monocytes. The occurrence of the pathological findings was more or less dependent on survival times.

Control liver grafts showed a monocyte, lymphocyte and lymphoblast infiltrate and to a lesser extent polymorphonuclear granulocytes, suggesting a combination of a vascular and cellular rejection. CsA-treatment in liver grafting resulted in a reduction in monocyte infiltration, compared to the untreated recipients. Extravasation of erythrocytes and infiltration of granulocytes was more pronounced. Slides of liver grafts in groups 7 and 8 revealed no infiltration except for one recipient, which survived 5 days. Morphological changes were not different as compared to the CsA-treated group.

Histological examination of the recipients that survived 9 and 10 days in group 9 unveiled similar but more prominent findings as seen in the CsA-treated liver recipients. The other animals demonstrated predominantly a severe mononuclear cell infiltrate.

Discussion

Clinically, liver allografts could successfully be transplanted across an ABO-barrier. This finding has initiated transplantation research to study the immuno-privileged position of the liver. To study this phenomenon in discordant liver grafting, GP to rat OLT has to be performed. The analysis of the technical feasibility of liver xenotransplantation has to be done by reproducing and comparing the results found by others. However, at the end of 1993, only two papers existed concerning GP to rat liver transplantation [8,9]. In concordant hamster to rat liver transplantation, reports are more plentiful. To study the technical feasibility of liver xenografting, we conducted the current experiments.

Experience in hamster to rat xenografting in our laboratory goes back to 1989 [10]. Over the years, immunosuppressive regimens have been optimised, finally resulting in long-term heart xenograft survival [11]. CsA- and TBI-treatment separately did not

result in long-term survival, whereas the combination resulted in survival times of more than 100 days [10,11]. The current experiments led to similar findings for the untreated and CsA-treated groups. However, TBI, a non-specific immunosuppressant, and the combination of CsA and TBI led to early death of most recipients. Nevertheless, survival times were prolonged in contrast to earlier findings [10].

Survival times of untreated and CsA-treated liver transplant recipients were similar as demonstrated by others [12-14]. However, application of the TBI and CsA/TBI immunosuppressant schedules resulted in a high incidence of early death of the recipients. These deaths were also observed in the other liver transplant groups. Besides the absence of liver grafting experience of the author, Yamaguchi et al. reported a 50% operative survival, indicating the influence of surgical and immunosuppressive trauma after liver xenografting [14]. Only one animal survived for 11 days, without signs of rejection, suggesting that the immunosuppressive therapy may be at least partially effective.

Comparison of survival times pointed out that liver grafts from untreated and CsA-treated recipients survived significantly longer than heart grafts. As hamster liver grafts seem to be as immunogenic as hamster heart grafts [15-17], this may imply that the liver is less susceptible to (antibody-mediated) rejection. Nevertheless, early deaths and failing immunosuppressive schedules preclude comparison of the TBI- and CsA/TBI-treated groups.

A fifth liver transplant group was added using FK506, a potent T-cell inhibitor. FK506 demonstrated partial long-term survival in concordant liver grafting, but was ineffective in heart grafting [4]. FK506 prolonged survival significantly, compared to untreated and CsA-treated recipients. The large variance seen in survival times is similar as previously found by others [4].

Unlike heart graft rejection, liver graft rejection is measured by recipient death. In the clinical situation, liver enzymes and proteins are measured upon decline of the health status. These parameters reveal an ongoing rejection, a venous congestion, or bile duct obstruction. Measurement of selective parameters revealed similar findings in liver xenograft recipients. As xenograft rejection is an extremely fast process, it can not be used as a conclusive tool for rejection. It can, however, function as a decisive tool in suspected early recipient death.

Therefore, although the surgical trauma and the additive immunosuppressive side-effects have a larger impact in liver xenografting than in heart grafting, hamster to rat liver xenotransplantation is technically feasible with similar results as found by others.

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CHAPTER TWO

Discordant liver transplantation in the guinea pig to rat model does not lead to classical hyperacute rejection

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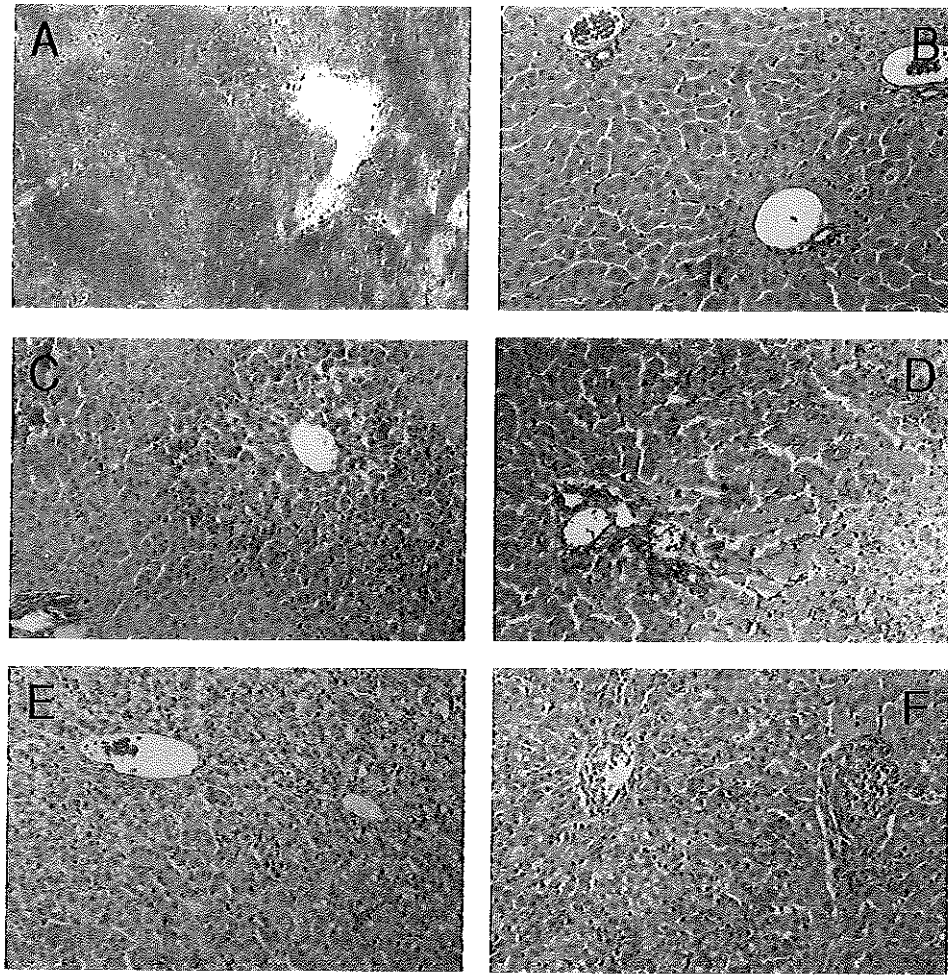

Figure 1. *Haematoxylin-eosine stained heart and liver graft sections.*
Hyperacutely rejected guinea pig (GP) heart (A). The typical characteristics of hyperacute rejection, like interstitial haemorrhage and oedema, are clearly seen. Non-rejected GP liver graft 1 hour (B), 4½ hours (C) and 18 hours (D) after grafting. Marked hydropic degeneration is demonstrated, especially 18 hours after transplantation. Non-rejected GP liver grafts of 99 hours (rat A) (E) and 147 hours (rat B) (F) after grafting. Severe hydropic degeneration and marginal infiltration is noticed. In all slides, no signs of haemorrhage or venular and capillary congestion were demonstrated. (amplifications: ×40)

Abstract

Discordant grafting, the best alternative for future transplantation, is hampered by hyperacute rejection (HAR). Yet, there might be a difference in susceptibility to HAR between organs. In allogeneic transplantation the liver is less sensitive to antibody-mediated rejection. In order to investigate whether this might also occur in discordant xenotransplantation, we performed orthotopic liver transplantation (OLT) from Dunkin Hartley guinea pigs (GP) to Brown Norway rats. Five groups were studied. In group 1, untreated controls survived for 1.5 to 4.5 hours (n=5). In order to investigate how long a recipient could survive without a functioning graft, animals in group 2 underwent total hepatectomy (tHx) with portal-caval shunt resulting in survival times from 2 to 7 hours (n=5). Antibody reduction by splenectomy (Spx) on day -5 (group 3) did not increase survival time (1 to 2 hours, n=5). Complement depletion by cobra venom factor (CVF) prolonged survival up to 35 hours (n=7, group 4). One animal lived for 4 days. The combined treatment of Spx and CVF resulted in similar survival times as following CVF alone, ranging from 2 hrs to 6 days (n=6, group 5). Surprisingly, none of the grafts in either of the groups showed classical signs of hyperacute rejection, like haemorrhage, oedema or obstruction of capillaries and veins as seen in the GP to rat heart transplantation model. Also liver enzyme parameters indicated no ongoing rejection. Immunohistochemistry revealed depositions of complement factors C1q, C3 and C6 on Kupffer cells but not on endothelial cells. These results indicate that, in this particular discordant model, the liver is not affected by the classical features of HAR. The beneficial effect of CVF on recipient survival, therefore, may be due to inhibition of a lethal secondary response evoked by the graft rather than to inhibition of HAR.

Introduction

In recent years, clinical organ transplantation showed constantly improving results, which led to an increasing number of patients awaiting a transplant procedure. Because, the number of organ donors did not increase accordingly, donor shortage has become a major problem. This rekindled a renewed interest in xenotransplantation over the last decennium. Grafting of tissue between closely related species showed promising results in animals [1-3] and has even been tried in man [4]. For various reasons the use of organs of species close to man can not be the ultimate solution to donor shortage so that organs of discordant species will have to be used. It is known that discordant transplantation results in hyperacute rejection (HAR), leading to destruction of the transplant within minutes to hours. Complement appears to play a pivotal role, either activated via the classical or alternative route, or both.

To study HAR in xenogeneic transplantation, the guinea pig (GP) to rat model is used frequently. This combination is representative for a discordant model as the rat has preformed xenoreactive natural antibodies (XNA) against the GP. However, lowering the preformed antibody level still results in HAR [5], suggesting that the alternative pathway is the major route of complement activation in this process [6]. From results obtained in the GP to rat heart transplantation model, it is known that rejection takes place within 1 hour. Complement depletion results in prolongation of survival times up to 3 days [7].

Clinically, it has been observed that livers, in contrast to other organs, can be transplanted across an ABO barrier and against T-cell cross-matches, which indicates that the liver is relatively insensitive to antibody-mediated rejection [8]. From recent studies we know that this also holds for the concordant hamster to rat model [3,9].

The aim of this study was to investigate whether this phenomenon of relative protection would also occur in the GP to rat orthotopic liver transplantation model.

Materials and methods

Animals

Female Dunkin Hartley GP (150-250 g) and male Brown Norway rats (250-300 g) were used as liver donors and recipients, respectively. The animals were purchased from Harlan C.P.B. (Austerlitz, The Netherlands). They were kept under controlled laboratory conditions and received food and tap water ad libitum. The "Committee on Animal Research" of the Erasmus University Rotterdam approved the experimental protocol.

Surgical procedure

Orthotopic liver transplantation (OLT) was performed according to Kamada et al. [10]. The donor was anaesthetised with urethane (Sigma, St. Louis, USA). The liver was isolated and the animals were anticoagulated with 75 I.U. heparin iv. (Organon Teknika B.V., Boxtel, The Netherlands). Five mg phentolamine mesilate (Ciba-Geigy, Basle, Switzerland) was injected iv. to accomplish vascular dilation in the liver. The supra- and infrahepatic vena cava (SVC, IVC) and portal vein (PV) were divided and the liver was flushed with 8 ml ice-cold University of Wisconsin solution. The liver was placed in a 4°C saline bath in which cuff preparations were carried out. The cuffs and splint were handmade from Teflon tubes (Medica B.V., 's Hertogenbosch, The Netherlands). The outside diameters of the Teflon cuffs were 2.3 mm and 2.1 mm for the PV and IVC, respectively. The everted veins were secured over the cuffs using a circumferential 8-0 nylon suture (B. Braun-SSC AG, Neuhausen am Rheinfall, Switzerland). The splint for bile duct had an outside diameter of 1.1 mm. The bile duct was secured by a circumferential 6-0 silk suture (B. Braun, Melsungen AG, Melsungen, Germany). Ether was used for induction and maintenance of anaesthesia in rats. The liver was removed after clamping the SVC, IVC, PV and superior mesenteric artery. The liver graft was placed orthotopically in the abdominal cavity. First, the SVC was anastomosed with a running 8-0 suture. Second, the PV-cuff was inserted in the PV of the recipient and secured with a circumferential 8-0 nylon and 6-0 silk suture. The bloodflow was

re-established by removing the clamps of the SVC, PV and artery. Next, the IVC and bile duct were anastomosed. After the operation, the recipients received 5 ml of warm glucose 5% with 0.1 ml of antibiotic procaine, penicillin G and dihydrostreptomycin (P.P.S) subcutaneously (Apharmo, Arnhem, The Netherlands). As a post-operative analgesic a bolus injection of 0.05 mg/kg buprenorphine (Reckitt & Colman, Hull, England) was given. The donor operation took about 50 minutes, the cuff preparation 15 minutes and the recipient procedure about 60 minutes. The anhepatic phase lasted about 20 minutes. This procedure resulted in the syngeneic rat to rat model in indefinite survival and in the concordant hamster to rat model in survival times up to 8 days (3, 4, 6, 6, 7, 7, 8, 8 days).

Splenectomy

In ether anaesthetised rats, the splenic vessels were coagulated using a bipolar forceps. The spleen was surgically removed.

Liver enzyme monitoring

Before and on day 3 and 5 after OLT, blood samples were taken. In the serum, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined as parameters for cellular damage. Alkaline phosphatase (AP), γ-glutamyl transpeptidase (γGT) were used as parameters indicating bile duct obstruction. Total bilirubin (tBIL) and cholinesterase (CHE) were measured as parameters for liver function.

Complement depletion and determination

Cobra venom (Naja naja kaouthia, lotnumber 57362) was obtained from ICN Pharmaceuticals B.V. (Zoetermeer, The Netherlands). Cobra venom factor purification procedure was performed according to Beukelman et al. [11]. One unit of CVF was designated as the amount of CVF causing 50% inhibition of lysis. Complement activity was measured in an endpoint lysis assay with sensitised sheep red blood cells as targets [12]. CH50-activity was expressed in units corresponding to the amount of serum causing 50% lysis.

Histology and immunohistochemistry

For evaluation of morphological changes, paraffin sections were stained with haematoxylin-eosine. Slides were scored for various parameters given in Table 2. The results were expressed as - (no histological changes) to +++ (severe histological changes).

For immunohistochemistry sections were stained by FITC-conjugated rabbit anti-mouse (RAM-FITC) with the following monoclonal antibodies: MARM-4 to demonstrate IgM and MARG2a-1 to demonstrate IgG2a (both obtained from Prof. Bazin, Brussels, Belgium). Complement depositions of C1q (classical pathway marker) were stained by FITC-conjugated goat anti-rabbit (Nordic Immunological Lab. BV., Tilburg, The Netherlands) after rabbit anti-rat C1q. Complement factor C3 was stained by polyclonal FITC-conjugated rabbit anti-rat IgG. Staining of complement factor C6 was based on the technique described by Raap et al. [13]. Liver sections from untreated rat and GP were taken as negative controls and hyperacutely rejected GP hearts as positive controls. The slides were analysed by fluorescence microscopy. Results were expressed as - (no depositions) to +++ (severe depositions).

Experimental design

Five groups were studied. Rats in group 1 (n=5) received no treatment. To evaluate how long a recipient could survive without a functioning liver, rats in group 2 (n=5) underwent total hepatectomy (tHx) and obtained a portal caval shunt. In group 3 (n=5) the recipients underwent Spx on day -5. Rats in group 4 (n=7) received a 1 ml bolus injection of CVF (9000 units/animal)

intravenously on day -1. Animals in group 5 (n=6) underwent Spx and received CVF on days -5 and -1, respectively. On day 0 the recipients (groups 1, 3, 4, 5) obtained an orthotopic GP liver graft. The endpoint of rejection was recipient death and survival was scored in hours. The upper liver lobe was removed and was processed for histology and immunohistochemistry.

Statistics

Statistical evaluation was performed using the logarithmic transformation values of the survival times. This was done to reduce the influence of outlying values. "One-way" analysis of variance (ANOVA) was performed on these data. If the ANOVA was significant on a 5% level, Duncan's multiple comparison test was carried out for possible differences among the means. The test was corrected for the fact that the comparisons were not statistically independent and for the unequal group sizes. Probability values lower than 0.05 were considered statistically significant. The grafts were scored as "functional" if they exceeded the upper 95% confidence interval limit of group 2 (8.7 hr).

Results

Efficacy of complement depletion

An immediate decline of complement levels was found after a single iv. bolus injection of 9000 U CVF. The levels were below detection limits on day 1. The complement-depleting effect of CVF lasted for more than 5 days, after which the complement gradually returned.

Complement levels of groups 1 and 3 were all normal at the time of liver grafting. Groups 4 and 5 demonstrated no CH50-activity at the moment of transplantation. Animals in groups 3 and 5 showed no difference in complement levels before and 5 days after Spx. The animal that survived for 147 hours had a complement level on day 5 that was 9.2 times higher than the level on day -5. Complement levels of GP liver donors were in the same range as in rats.

Efficacy of xenogeneic natural antibody depletion

XNA levels were measured on day -5 and the day of transplantation. Five days after Spx, XNA levels were significantly decreased to a mean of 34.5% ($p=0.008$). No statistical difference could be demonstrated between the reductions in XNA levels in groups 3 and 5.

Graft survival

Graft survival, as noted in Table 1, showed a highly significant difference between the group means ($p=0.0000$, $s_{res}=0.868$). The difference between the survival times of rats that underwent tHx (group 2) and the untreated controls (group 1) was not statistically significant. The addition of Spx to the OLT (group 3) did not prolong the survival of the recipient. CVF alone (group 4) prolonged the survival significantly when compared to the tHx-group ($p<0.01$), the untreated controls ($p<0.01$) and the splenectomised rats

Table 1. *Survival times following discordant liver transplantation.*[a]

Group	Treatment	Survival (hours)	Geometric mean	95% Confidence Interval
1	Untreated	1½, 1½, 2, 2, 4½	2.1	0.9-4.7
2	tHx	2, 4, 4, 4, 7	3.9	1.7-8.7
3	Spx	1, 1½, 1½, 2, 2	1.6	0.7-3.5
4	CVF	16, 18, 18, 20, 20½, 35, 99	25.7	13.0-50.6
5	Spx + CVF	2, 3½, 4, 12, 29, 147	10.6	5.1-22.1

[a]Livers in groups 1, 3, 4 and 5 were transplanted from GP to BN rats. Group 2: total hepatectomy (tHx), group 3: splenectomy (Spx) on day -5, group 4: cobra venom factor (CVF) on day -1, group 5: combination of Spx and CVF. No significant differences exist between groups 1, 2 and 3. Groups 4 and 5 are statistically different from groups 1 and 3.

($p<0.01$). The combination therapy (group 5) showed a significantly extended survival compared to the untreated controls ($p<0.01$) and the splenectomised rats ($p<0.01$). There was no statistical difference between group 4 and group 5.

Reperfusion of the liver grafts in groups 1 and 3 was inhomogeneous with parts remaining inadequately perfused. In the CVF-treated groups, this recolouration problem was reduced but still present. Most of the rats died showing the clinical signs of shock.

Histology

Specimens for histological examination were obtained from the transplanted livers after death of the animals. It appeared that the occurrence of pathological features were more dependent on survival time than on the various treatment modalities (see Table 2). Histology of the recipients, who survived between 1 to 4½ hours, showed marked hydropic degeneration, but this was scored as "reversible" (see Figure 1B,C). No necrosis or infiltration was seen. The livers that survived for more than 4½ hours presented severe hydropic degeneration (see Figure 1D). Signs of local necrosis in the lobules were observed, but no infiltration was seen. In the long surviving rats A and B, these changes were of the same magnitude (see Figure 1E, F). In addition, there was some infiltration in the lobules. None of the animals showed interstitial haemorrhage, oedema and capillary and venular congestion (see Figure 1A).

Table 2. *Histology of "rejected" liver grafts according to recipient survival time.*[a]

Survival times (hours)	Hydropic degeneration	Necrosis	Infiltration
1 to 4½	++	-	-
4½ to 35	+++	+	-
99	+++	+	+
147	+++	+	+

[a]Scores varied from - (no histological changes) to +++ (severe histological changes).

Immunohistochemistry

Immunohistochemistry revealed treatment-dependent and survival time dependent depositions of IgM and IgG (see Table 3). In the untreated control group modest depositions of IgM and IgG were found in the liver vessels. This was less pronounced in the CVF-treated group. In rat A, IgM depositions were more present in the lobules than in the vasculature. Spx resulted in an almost complete absence of antibody depositions in livers of short surviving animals. The prolonged surviving rat B showed massive depositions of IgM in the lobules and of IgG in the lobules and vasculature.

Depositions of complement factors C1q, C3 and C6 were found on Kupffer cells but not on endothelial cells in the sinusoids. The depositions were less pronounced than in hyperacutely rejected GP hearts, which served as positive controls (see Table 4). The livers of groups 1 and 3 showed more depositions of C3 than the CVF-treated animals. The two long-surviving animals had only slight depositions of C6 on the Kupffer cells.

Liver enzyme monitoring

One rat in group 4 (rat A) and one in group 5 (rat B) survived for more than 4 days. In these animals, liver enzyme levels were measured (see Figure 2). There was a 1.3- to 4-fold increase in ASAT- and AP-levels in both animals. After transplantation

Table 3. *Depositions of IgM and IgG in vasculature and lobules of liver transplants.*[a]

Group	Treatment	IgM deposition		IgG deposition	
		Vessels	Lobules	Vessels	Lobules
1	Untreated	+	-	++	-
3	Spx	-	-	-	-
4	CVF	±	+	±	±
5	Spx + CVF	-	+	+	+

[a]Scores varied from - (no depositions) to +++ (massive depositions).

Table 4. *Depositions of complement factors C1q, C3 and C6 on Kupffer cells in liver grafts.*[a]

Group	Treatment	C1q	C3	C6
1	Untreated	+	++	±
3	Spx	+	++	±
4	CVF	±	±	±
5	Spx	±	±	±
Positive controls[b]		+++	+++	+++

[a]Scores varied from - (no depositions) to +++ (massive depositions).

[b]Hyperacutely rejected guinea pig hearts.

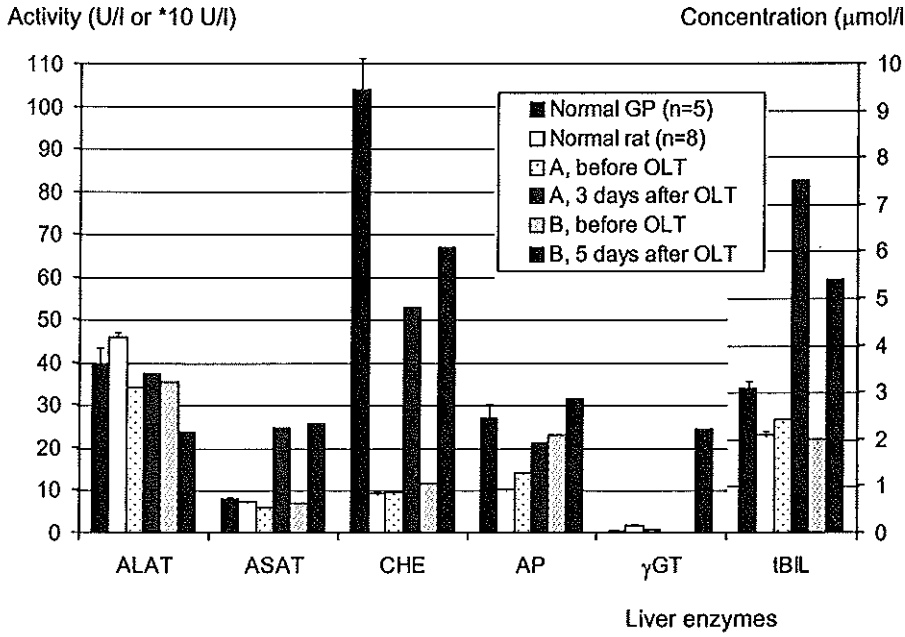

Figure 2. *Liver enzyme levels in normal guinea pigs, normal rats and two long surviving rats, A and B, at various days after liver xenografting.*

ALAT- and γGT-levels are displayed in U/l, ASAT-, CHE-, and AP-activity in *10 U/l, and tBIL in µmol/l. ALAT: alanine aminotransferase, ASAT: aspartate aminotransferase, CHE: choline esterase, AP: alkaline phosphatase, γGT: γ-glutamyl transpeptidase, tBIL: total bilirubin, GP: guinea pig.

CHE-activity increased 5.5 to 7 times, compared to pre-transplant activity. Normal CHE-activity in GP is about 10 times higher than in rats. In rat B, the γGT-level rose from zero to 25 U/l, but was accompanied by a, for rats, clinically irrelevant increase of bilirubin level in serum.

Discussion

HAR of hearts in the GP to rat model takes place within several minutes after reperfusion [7]. Animal survival times as observed in the current study (groups 1 and 3) suggest that HAR also occurs in our model. In general, HAR is directed against the endothelium of the transplanted organ. Activation of endothelium by the complement system and/or antibodies causes triggering of metabolic changes [14]. In the heart transplantation model, histologically, this process gives rise to interstitial haemorrhage, oedema, capillary and venular congestion but hardly any cellular infiltration [15]. We noticed similar features in our hamster to rat liver transplantation model following

donor specific sensitisation (unpublished results). This contrasts with our current results in the discordant liver transplant model. In none of the animals of the various experimental groups, haemorrhage or obstruction of capillaries and veins was demonstrable. These results are supported by other reports on discordant liver grafting [2,16]. Crafa et al. showed that discordant livers displayed no or only low intensity of the above-mentioned parameters [16]. Gridelli et al. found diffuse mild oedema, sinusoidal dilatation and some hydropic degeneration [2]. Settaf et al., however, noted extravasation of blood into the parenchyma and congestion of the portal vein in 8 out of 14 liver recipients [17]. In our series, hydropic degeneration was the only pronounced morphological change that did emerge. The severity of this degeneration increased with survival time, leading to slight necrosis and infiltration around the veins. No signs of the classical HAR were seen.

It can be argued that the necrosis seen in the livers was caused by ischaemia, due to the fact that the hepatic artery of the graft was not anastomosed. However, this seems unlikely since the local necrotic fields were not situated around arteries. Earlier reports have demonstrated that the hepatic artery is not necessary for the survival of the graft, but that absence of the arterial blood supply may affect the bile duct system [18]. It is more likely that ischaemia due to poor reperfusion of the GP livers was a cause of the observed necrosis. Remarkably in the long-term surviving liver grafts, monitoring of liver enzymes did not reveal the occurrence of rejection. ALAT-levels were not changed and the ASAT-levels were only slightly elevated. The elevated CHE-levels indicated that the liver in the recipient functioned like a GP liver. Such increased CHE-levels were never seen in our concordant liver grafting model (unpublished results). To assess the role of XNA on graft survival, antibody reduction by Spx was performed. Removal of 50% to 90% of the circulating preformed antibodies had no effect. Immunohistochemistry revealed no IgM- or IgG-depositions in most of the transplanted livers of splenectomised rats. Still, the survival times of splenectomised rats were not prolonged. Therefore, it seems likely that a contribution of the classical pathway of complement activation is unimportant in our model.

This reasoning, combined with the prolonged survival times obtained with CVF, strongly suggests a dominant role of the alternative pathway of complement activation in our liver model. It agrees with earlier findings in the GP to rat heart transplantation model [6,19], with one important difference: the classical signs of HAR were never seen in our experiments. In addition, immunohistochemistry revealed only depositions of C1q, C3 and C6 on Kupffer cells and not endothelial cells, which are considered to be the main targets of hyperacute rejection [14].

Complement levels remained undetectable in representative, CVF-treated control animals for at least 5 days. Interestingly, long-surviving rat B showed marked serum

complement activity on day 5. This activity was 9 times higher than the level on day -5. Because the liver is the main source of complement, it is conceivable that the complement was of donor origin. Consequently, it is possible that rats died because of a graft vs. host reaction via the release of complement of the grafted liver. However, Braidley et al. demonstrated elegantly that HAR does not occur in the rat to GP model [20], which makes the "melt-down"-theory less likely.

The question remains why our rats died so early with an apparently functioning, non-rejected liver graft. Gridelli et al. showed that only 5 minutes after reperfusion of liver xenografts, the lumen of sinusoids was filled with amorphic material and cellular debris [21]. This debris came from endothelial cells and was not observed in the allogeneic situation. It is possible that endothelial cell activation also occurred in our model, leading to the release of large amounts of vasoactive substances causing irreversible shock, without signs of classical HAR. In addition, Kupffer cells might have been activated by ischaemia, reperfusion and complement activation provoked by grafting, which could lead to lethal levels of TNFα and superoxide [22,23]. This shock-hypothesis should include an important role for complement, because treatment with CVF did result in prolonged survival. The role of activated complement in evoking shock has been mentioned earlier [24].

Therefore, our current studies are focussed on the analysis of the possible role of GP Kupffer cells and cytokines in the induction of shock by liver transplantation.

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CHAPTER Three

Complement C6 and C2 biosynthesis in syngeneic PVG/c$^-$ and PVG/c$^+$ rat strains – The PVG/c$^-$ rat as a recipient in xenotransplantation

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Abstract

In the discordant guinea pig to rat xenotransplantation model, heart grafts reject within one hour. Liver graft recipients, however, die with apparently functioning, non-rejecting transplants. No classical signs of hyperacute rejection are seen. Even after complement depletion with cobra venom factor (CVF), early recipient death is noticed. As CVF-toxicity was suspected to cause this phenomenon, liver xenografting in the C6 deficient and partially C2 deficient PVG/c$^-$ rat strain may result in prolonged survival. Therefore, the complement deficiency of the PVG/c$^-$ rat and its suitability for transplantation was further analysed.

Livers of PVG/c$^-$ rats were transplanted in syngeneic, 'complement-sufficient' PVG/c$^+$ rats (Tx-L) and visa versa (Tx+L). The C6 and C2 levels in Tx-L rats declined within 2 days to 25% and 30%, respectively, and remained stable for more than 6 weeks. In Tx+L rats, an initial increase with maximum C6 levels of 119% at day 10 was followed by a gradual decrease. Finally, C6 was no longer detectable 28 days after transplantation. This decline in C6 levels was dependent on antibody production against C6. The C2 level reduction remained unexplained. No significant change in the C3, C4, factor H and factor B levels was observed. Expression of C6 mRNA in the grafted PVG/c$^+$ liver was comparable to the expression of C6 mRNA in control PVG/c$^+$ livers. C6 mRNA expression in the transplanted PVG/c$^-$ liver and the control PVG/c$^-$ liver was lower. In conclusion, PVG/c$^-$ rats seem suitable for liver transplantation. Antibodies cause the deficiency of C6, but do not explain the partial C2 depletion. It is expected that upon discordant grafting complement activation still occurs, although the formation of the terminal complement complex is inhibited. Therefore, xenograft rejection via the anaphylatoxins can not be excluded.

Introduction

Human organ transplantation has developed itself to a rather successful technique. A disadvantageous effect of this process was that waiting lists came into existence. Even in highly developed countries, patients die awaiting transplantation. Xenotransplantation, grafting of organs between different species, might be a possible solution to this problem. Considerations, regarding ethics, pathology and farming, indicated that the pig is the best possible organ donor. However, rejection of these discordant organs is rather vigorous. Hyperacute rejection (HAR) ensues, resulting in destruction of the graft by complement activation within minutes to hours. The classical route, initiated by preformed xenoreactive natural antibodies, and alternative route of complement activation both play pivotal roles.

To study this process, the guinea pig (GP) to rat model is often used. The rat has preformed xenoreactive natural antibodies against the GP. Nevertheless, the alternative pathway is the major route of complement activation [1,2]. GP heart to rat transplantation results in rejection within 1 hr, indicating HAR. Complement depletion prolongs heart graft survival up to 3 days [3]. However, discordant liver grafting did not show classical signs of HAR, like haemorrhage, oedema or obstruction of capillaries and veins as seen after GP to rat heart grafting [4]. Although complement depletion by cobra venom factor (CVF) prolonged graft survival, the recipients died with an apparently functioning, non-rejecting liver transplant. Other intriguing findings in experiments that include CVF-treatment have been reported [5]. Also, "hydropic-degeneration"-like symptoms have been seen in normal rat livers after administration of CVF, in principle a non-toxic complement depleting glycoprotein in cobra venom (Dr. Scheringa, personal communication). It could well be that a potential liver toxicity of CVF prevents prolongation of discordant hepatic graft survival.

In 1994, a PVG/c rat strain was described with a deficiency for complement C6 and a partial deficiency for C2 [6]. The GP to PVG/c$^-$ rat liver transplantation model may elucidate the potential toxic role of CVF. Therefore, in the current study the PVG/c$^-$ rats were analysed for their status of complement deficiency and suitability for liver transplantation.

Materials and methods

Animals

Three-month-old male complement-deficient PVG/c rats (PVG/c$^-$) (Bantin and Kingman, Edmonds, UK) and complement sufficient PVG/c rats (PVG/c$^+$) (Harlan C.P.B., Austerlitz, The Netherlands), weighing 200-300 grams were used for the experiments. The animals were housed in an accredited animal breeding facility with free access to normal rat chow and water. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). The specific protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands.

Procedure liver transplantation

Orthotopic liver transplantation (OLT) was performed according to Kamada et al. with some minor modifications [4,7]. Syngeneic liver transplantations were performed using Teflon cuffs of 3.3 mm and 2.1 mm for the infrahepatic vena cava and portal vein, respectively. A 0.63 mm splint was applied to connect the bile ducts. The donor operation took about 45 minutes, the cuff preparation 15 minutes, and the recipient about 60 minutes. The anhepatic phase did not exceed 25 minutes. Recipient were sacrificed 7 weeks after transplantation.

Histology

At week 7, necropsy was performed. The upper liver lobe was removed and processed for histology. Formalin fixed tissue was paraffin embedded, cut and periodic acid-Schiff stained for examination of morphological changes by conventional light microscopy.

Haemolytic assays

Haemolytic assays for C3, CH50 and C2 were performed as described by Daha and van Es and Brandt et al. [8,9]. The haemolytic activity of C6 was assessed using sheep red blood cells sensitised with sub-agglutinating amounts of rabbit IgG anti-sheep erythrocytes (EA) and C6-deficient human serum. Briefly, rat serum samples were diluted in half-isotonic Veronal buffered saline ($DGVB^{++}$; 0.05% gelatine, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 3% dextrose). One hundred microliters were incubated with 100 µl EA in $DGVB^{++}$ (10^8 cells/ml), containing a 1/100 dilution of C6-deficient human serum, for 1 hr at 37°C. The samples were centrifuged for 10 min at 2,000 rpm and haemolysis was measured in the supernatants with a Beckman photospectrometer at absorbance of 414 nm. The number of effective haemolytic sites generated (Z) was calculated and expressed as percentage consumption of complement relative to haemolytic activity in PVG/c^+ serum before transplantation. The consumption in PVG/c^+ serum was assigned the value of 100%.

Rat C6 sandwich ELISA

Wells in 96-wells microtiter plates were incubated for 2 hr at 37°C with 100 µl monoclonal mouse anti-rat C6 (1 µg/ml) in 0.1 M carbonate buffer (pH 9.6). Subsequently, the plates were incubated with serum samples, diluted 1/50 and 1/100 in PBS containing 0.05% Tween and 2% casein (PTC) for 1 hr at 37°C. Rat C6 was detected by incubating the plates with DIG-conjugated affinity-purified polyclonal rat anti-rat C6 antibodies for 1 hr at 37°C. Finally, the plates were incubated with HRP-labelled anti-DIG Fab for 1 hr at 37°C and with the HRP-substrate, ABTS. The reaction was terminated after 30 min with 2% oxalic acid. The amount of substrate generated was determined using a Titertek Multiskan plate reader at optical density of 415 nm. Between incubation steps, the plates were washed with PBS-Tween and the antibodies were diluted in PTC. As a standard, serial dilutions of pooled normal Wistar serum were assayed at the same time. Because the haemolytic activities (U/ml) of rat and human C6 were identical, it was assumed that rat C6 concentrations were comparable to the concentration of approximately 60 µg/ml found in human serum [10]. Therefore, 60 µg/ml was used as estimated C6 concentration in rat serum.

Detection of anti-rat C6 antibodies in Tx+L serum

Wells in 96-wells microtitre plates were incubated for 2 hr at 37°C with 100 µl monoclonal mouse anti-rat C6 (1 µg/ml). Subsequently, the plates were incubated with PVG/c^+ serum, diluted 1/40 in PTC for 1 hr at 37°C. Sera of Tx+L rats, obtained at various time points after transplantation, were diluted 1/200 and 1/1000 in PTC and the plates were incubated with these dilutions for 1 hr at 37°C. As a control the dilutions were tested on the mouse monoclonal coating alone and the plates were also incubated with PVG/c^- serum as a negative control. Sera of PVG/c^- rats immunised with PVG/c^+ serum were used as a positive control. Finally, the plates were incubated with PTC-diluted biotin-conjugated mouse monoclonal anti-rat κ-chain for 1 hr at 37°C, followed by incubation with STREP-HRP, diluted in PTC, for 1 hr at 37°C. Bound antibodies were detected by incubating the plates for 30 min with 2% oxalic acid.

Northern blot and dot blot analysis

Total RNA from liver tissue was isolated and purified as described by Timmerman et al. [11]. Thirty micrograms of total RNA was separated on a formaldehyde containing 1% agarose gel and blotted onto nitro-cellulose filters. Gel electrophoresis, RNA transfer and high stringent hybridisation were performed as described by Timmerman et al. [11,12]. Forty micrograms of total RNA and 3-fold serial dilutions were spotted on a nitrocellulose filter using a Bio-dot apparatus [12]. The cDNA probes were labelled with [α-^{32}P]dCTP by random priming labelling [13]. The probes used were a 490 bp rat C6 cDNA fragment and a 1300 bp EcoRI GAPDH cDNA fragment to quantify the amount of RNA loaded per lane. The 490 bp rat C6 cDNA clone was isolated from a rat liver cDNA library in λgt10 (RL1020a, Clonetech Laboratories, Palo Alto, CA, USA). The sequence of the 490 bp of the rat C6 cDNA was determined by dideoxy-sequencing and sequence analysis was performed using Geneworks (IntelliGenetics, Mountain View, CA, USA). Rat C6 was found to be homologous to human C6 at DNA and at protein level.

Experimental design

Livers were transplanted from PVG/c$^-$ to PVG/c$^+$ (Tx-L) and visa versa (Tx+L) (n=3, both). No treatment was given. Blood samples were obtained at days 1, 2, 4, 6, 8, 11, 14, 21, 28, 35 and 42. The sera were tested by haemolytic assays for C2, C3, C6, CH50 and a specific rat anti-rat C6 ELISA. For the assessment of hepatic mRNA expression, rats were sacrificed and liver tissue biopsies were stored at -80°C until use. A part of the upper liver lobe was processed for histology.

Results

Liver transplantation and histology

The recipient PVG/c$^-$ rat could tolerate the liver transplant procedure and with the PVG/c$^+$ rat as liver donor survived for more than 6 weeks. After initial weight loss, it slowly returned in 2 to 3 weeks to its initial value. At necropsy, histology showed no morphological changes compared to liver sections from PVG/c$^-$ and PVG/c$^+$ rats.

Haemolytic activity

Haemolytic activity of complement was determined in serum samples of both donor and recipient rats before transplantation and during 6 weeks after OLT. Assessment of the

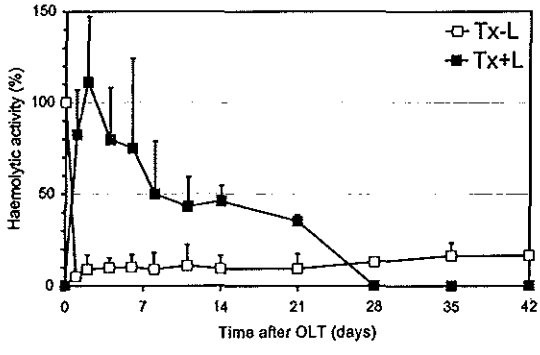

Figure 1. *Complement activity (CH50) in Tx-L and Tx+L rats.*
Complement-deficient (Tx-L, n=3) and complement-sufficient livers (Tx+L, n=3) were transplanted to complement-sufficient and -deficient rats, respectively. CH50 activity is expressed as percentage ± SD of the PVG/c$^+$ rat CH50 before transplantation.

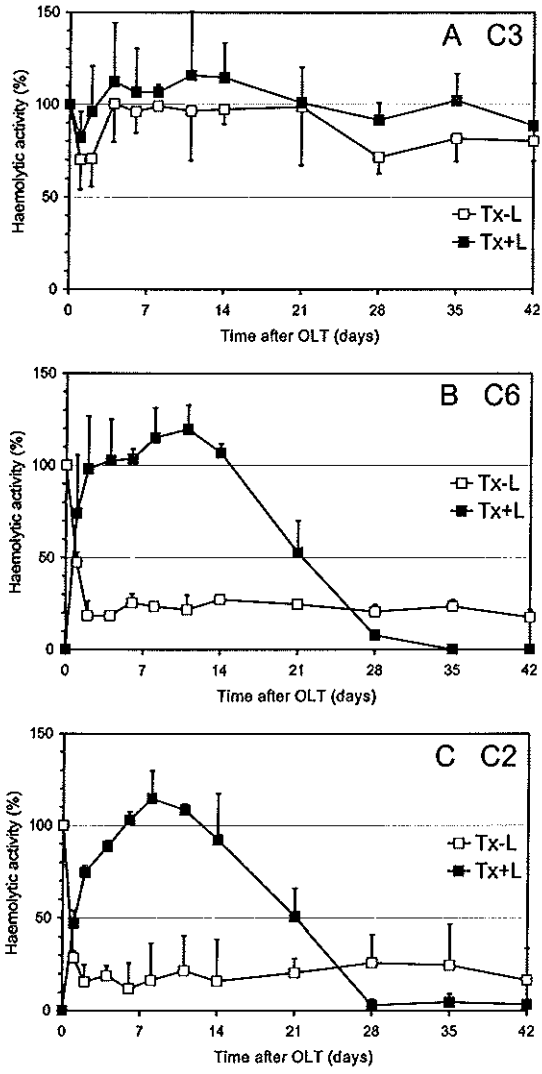

Figure 2. *Circulating C3, C6 and C2 levels in Tx-L and Tx+L rats determined by haemolytic assay.*
Complement-deficient (Tx-L, n=3) and complement-sufficient livers (Tx+L, n=3) were transplanted to complement-sufficient and -deficient rats, respectively. C3 (A), C6 (B) and C2 (C) haemolytic activities are expressed as a percentage ± SD of haemolytic activity of the PVG/c$^+$ rat before transplantation.

CH50 haemolytic activity in Tx-L and Tx+L sera showed a decline to 15% after 2 days after OLT. It remained at approximately the same level for more than 6 weeks. In Tx+L sera, an initial increase in the CH50 levels was found, reaching maximal levels of 111% two days after transplantation. Surprisingly, these levels declined gradually and 28 days following transplantation, the CH50 activity was no longer detectable (see Figure 1).

Functionality of the liver grafts was determined by the haemolytic activity of C3. No significant change in C3 haemolytic activity was observed after liver transplantation for both the Tx-L and Tx+L rats (see Figure 2A). In sera of Tx-L rats, the haemolytic activities of C6 and C2 were approximately 25% and 30%, respectively, as compared to haemolytic activity in sera of PVG/c$^+$ rats (see Figure 2B, C). In Tx+L sera, an initial increase in the C6 and C2 haemolytic activity was observed, which reached maximal levels of 119% and 114%, respectively, ten days after transplantation. Surprisingly, the haemolytic activity of C6 decreased during the next 20 days of follow-up and ultimately no C6 activity could be detected. Circulating C2 levels also declined to levels of 5% of

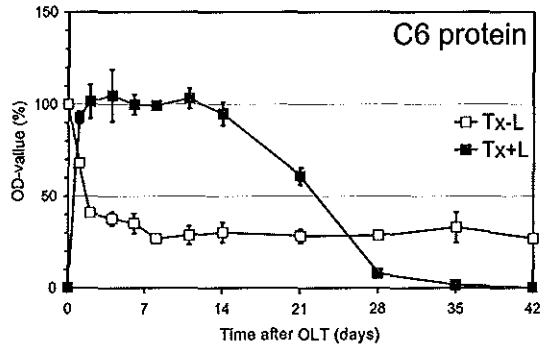

Figure 3. *Circulating C6 levels in Tx-L and Tx+L rats determined by ELISA.*
Complement-deficient (Tx-L, n=3) and complement-sufficient liver (Tx+L, n=3) were transplanted to complement-sufficient and -deficient rats, respectively. The C6 levels are expressed as a percentage ± SD of optical density of the PVG/c⁺ rat before transplantation.

the levels normally found in PVG/c⁺ serum. C6 antigenic levels as measured by C6-specific ELISA showed similar changes as observed for haemolytic C6 (see Figure 3). Measurements of other complement components (C4, factor B and factor H) in sera of Tx-L and Tx+L rats were found to be comparable to the level found in normal PVG/c⁺ and PVG/c⁻ rats [6]. No decline of these components was seen in Tx+L rats.

Haemolytic activities of hepatic and extrahepatic C6

Brauer et al. already showed that the molecular weights of hepatically and extrahepatically synthesised C6 were identical [14]. Haemolytic activity of C6 was measured in sera of PVG/c⁺, Tx-L and Tx+L rats together with antigenic C6, as measured by ELISA. It was found that hepatic C6 (Tx+L) had a functional activity of 94 U/μg and the activity of extrahepatic C6 (Tx-L) was 90 U/μg, indicating no differences in haemolytic activity between hepatic and extrahepatic C6 (see Table 1).

Detection of anti-rat C6 antibodies

Prior to transplantation, no anti-rat C6 response was detectable in sera of Tx+L rats (results not shown). On day 28 after transplantation, a positive reaction against PVG/c⁺ serum, and thus C6, was observed in sera of Tx+L rats, while no response was found in

Table 1. *The haemolytic activity (U/μg) of C6 in the circulation of PVG/c⁻, PVG/c⁺, Tx-L and Tx+L rats.*

Strain/recipient	U/ml	Rat C6 concentration (μg/ml)	U/μg
PVG/c⁻	ND[a]	ND	ND
PVG/c⁺	4832 ± 1221	55	88 ± 22
Tx-L[b]	568 ± 23	6.25	90 ± 4
Tx+L[b]	4992 ± 181	53	94 ± 4

[a]ND: not detected.

[b]Sera obtained 8 days after transplantation were used to determine the haemolytic activity.

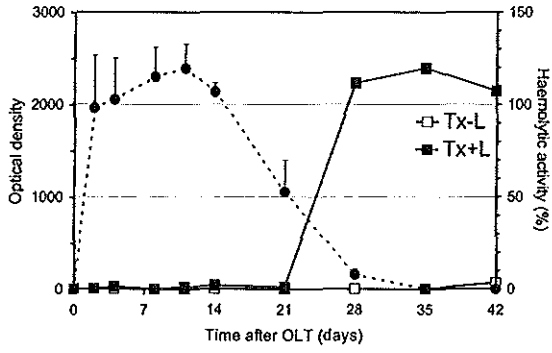

Figure 4. *Anti-rat C6 antibodies in serum of Tx+L rats.*

Sera of a Tx-L and a Tx+L rat were tested for the presence of anti-rat C6 antibodies in a specific ELISA. On the left y-axis the optical density assessed by ELISA is depicted. For the other Tx+L and Tx-L rats similar results were obtained. The C6 haemolytic activity in Tx+L rats (--●--) is expressed as a percentage ± SD of haemolytic activity of the PVG/c$^+$ rat before transplantation (right y-axis).

serum of Tx-L rats (see Figure 4). The response against rat C6 was still present 42 days after transplantation. The C6 haemolytic activity in sera of Tx+L rats is also depicted.

Hepatic mRNA expression

Northern blotting of 40 μg liver-isolated mRNA demonstrated a specific band for both PVG/c$^-$ and PVG/c$^+$ rat livers, indicating C6 mRNA expression (see Figure 5). The dot blot analysis of 3-fold serial dilutions of 40 μg mRNA from PVG/c$^-$, PVG/c$^+$, Tx-L and Tx+L rats showed comparable steady state mRNA levels in the PVG/c$^+$ and Tx+L livers (see Figure 5). In PVG/c$^-$ and Tx-L liver, the C6 mRNA expression is comparable but 7-fold lower than the expression found in PVG/c$^+$ and Tx+L livers.

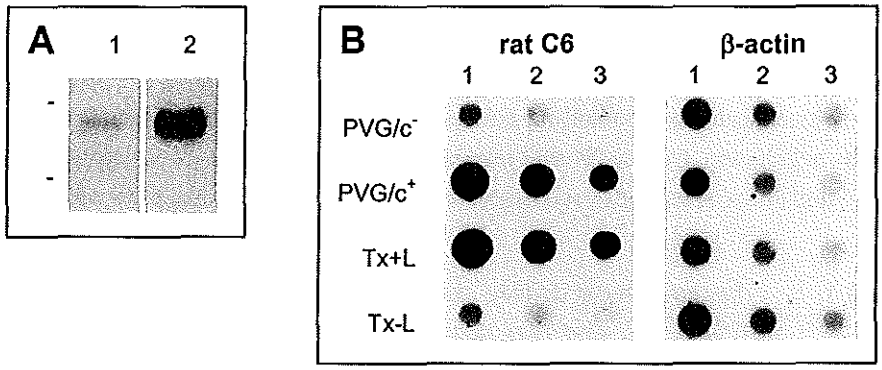

Figure 5. *Expression of C6 mRNA in livers of PVG/c-, PVG/c+, Tx+L and Tx-L rats.*

(A) Northern blot analysis with lane 1, 40 μg PVG/c- liver and lane 2, 40 μg PVG/c+ liver mRNA. After electrophoresis and blotting, the filter was hybridised with a rat specific C6 cDNA probe. (B) A total of 40 μg mRNA was isolated from livers of PVG/c$^-$, PVG/c$^+$, Tx+L and Tx-L rats and spotted in 3-fold serial dilutions. The dot blot was hybridised with a rat specific C6 cDNA probe and as a control hybridised with a GAPDH cDNA probe.

Discussion

Although complement depletion by CVF prolonged discordant liver graft survival, recipients died after several hours to days. Histologically, liver grafts of these animals demonstrated no signs of rejection and were apparently functional. Administration of CVF to naive rats showed (reversible) microscopical lesions in the livers (Dr. Scheringa, personal communication). The search for other than CVF-depleted complement deficient rats to test this 'CVF-toxicity' hypothesis led to the recently described PVG/c$^-$ rat [6]. To test the suitability of the PVG/c$^-$ rat for the complex life-supporting liver transplantation and the analysis of the status of complement deficiency, the present study was conducted.

The 'syngeneic' liver transplant procedure was well accepted by the PVG/c$^-$ rat after tuning of the technique to meet the model specific requests. Compared to the BN rat, recovery of the PVG/c$^-$ rat was slower, as measured by weight gain. Nevertheless, no other complications were noted.

The PVG/c$^-$ rat was described by Leenaerts et al. [6]. To further elucidate the complement-deficient state of this strain, the synthesis of C6 and C2 was studied by performing liver transplantations between a complement-deficient (PVG/c$^-$) and 'complement-sufficient' (PVG/c$^+$) rat strain. The circulating C6 and C2 levels in PVG/c$^-$ rats are not detectable and 5%, respectively, as compared to circulating levels of the proteins in PVG/c$^+$ rat [6,9]. In PVG/c$^+$ rats grafted with a PVG/c$^-$ liver (Tx-L), the circulating levels of C6 and C2 were 25% and 30%, respectively, as compared to PVG/c$^+$ rats. The C6 levels were higher than the levels normally found in PVG/c$^-$ rats. Since the liver is not the source of C6 in Tx-L rats and the half-life was calculated to be 12 hr, C6 found in the circulation of Tx-L rats was from extrahepatic origin. Furthermore, we found the haemolytic activity of extrahepatically and hepatically synthesised C6 was similar.

Transplantation of PVG/c$^+$ livers to PVG/c$^-$ rats (Tx+L) revealed the contribution of hepatically synthesised C6 and C2 to circulating levels of C6 and C2. Surprisingly, after an initial increase, the C6 and C2 levels gradually declined. The antigenic C6 levels followed a similar course, suggesting that C6 synthesis was suppressed by the C6 sufficient liver in Tx+L rats or that C6 was cleared from the circulation due to the formation of anti-rat C6 antibodies. Brauer et al. also performed transplantations of PVG/c$^+$ liver to PVG/c$^-$ rats [15]. The authors found that the C6 levels in these rats were 80% as compared to the levels in PVG/c$^+$ rats in the first 14 days following transplantation [15]. In these rats, C6 was also no longer detectable 28 days after transplantation [15]. It was hypothesised that the decline in C6 levels may be due to antibodies against C6, which are generated in the originally C6 deficient recipients [15].

In the present study, anti-rat C6 antibodies were formed in PVG/c$^-$ rats grafted with PVG/c$^+$ livers. The titre of these antibodies in Tx+L serum increased at the same time (day 28) that C6 levels in the circulation of these rats started to decline. Therefore, the disappearance of rat C6 from the circulation of Tx+L rats may be explained by neutralisation and subsequent clearance of rat C6.

Another interesting phenomenon is that the kinetics of C6 and C2 were similar in both Tx-L and Tx+L rats. This suggests that the synthesis of C6 and C2 in PVG/c$^+$ and PVG/c$^-$ rats is somehow connected. However, the antibody-hypothesis for C2 is not favoured by the finding that the levels of C2 were only lowered. The presence of C2 antigens in PVG/c$^-$ rats prior to transplantation makes it unlikely that antibodies against C2 are generated in the recipients. However, one may argue that antibodies against C2 are formed in Tx+L rats which recognise epitopes on C2, synthesised by the PVG/c$^+$ liver, which were previously not exposed on C2 synthesised in the PVG/c$^-$ rat. Unfortunately, we were not able to test this hypothesis because purified rat C2 and specific anti-rat C2 antibodies were not available. An alternative explanation for the reduction in C2 levels is that immunecomplexes of C6 and anti-rat C6 might activate the classical pathway and induce a secondary deficiency of C2. However, no change was observed in C1q levels, suggesting that significant classical pathway activation was absent.

The decline in C6 and C2 levels in Tx+L rats was not attributable to a dysfunctional liver concerning its production capacity. Levels of C3, C4 and factor B, components mainly synthesised in the liver, were not changed either, indicating normal synthesising ability of the transplanted livers [16-18]. Although the circulating C6 levels were reduced in Tx+L rats, the hepatic C6 mRNA expression in these rats was comparable to the C6 mRNA expression in livers of PVG/c$^+$ rats. The fact that normal C6 mRNA levels were detected in the grafted PVG/c$^+$ liver suggests that the reduction in C6 levels resulted from the formation of anti-rat C6 antibodies.

In conclusion, the PVG/c$^-$ rat seems a suitable recipient in discordant liver grafting. As the alternative pathway is the major route of complement activation in the GP to rat transplantation model [2], no additional effect of the C2 deficiency is expected. However, the deficiency of C6 may totally inhibit the formation of the effector of the complement cascade, the terminal complement complex. Nevertheless, activation of the complement system still occurs, leading to the formation of inflammatory mediators, like C3a and C5a. Therefore, graft rejection via the anaphylatoxins can not be excluded.

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CHAPTER Four

Ischaemia/reperfusion injury and surgical trauma preclude the use of the guinea pig in discordant liver grafting

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Abstract

After xenotransplantation of guinea pig (GP) heart into rat recipients, grafts are hyperacutely rejected. Depletion of complement by cobra venom factor (CVF) leads to prolongation of survival. GP to rat liver grafting, however, results in early recipient death without hyperacute rejection. CVF-toxicity or primary non-function (PNF) of the GP liver might cause this phenomenon.

To study CVF-toxicity, GP heart and livers were transplanted in C6 deficient PVG rats (PVG/c$^-$). Control and CVF-treated heart graft recipients survived 1.7 and 3.7 days, respectively. However, 40% of the recipients died without a rejected graft. All liver grafted PVG/c$^-$ rats died after several hours. Histology indicated no ongoing rejection.

To investigate possible PNF, partial (30%) GP livers were grafted in 70% hepatectomised BN and PVG/c$^-$ rats. BN rat survival times after CVF treatment (mean 30.9 hours) were similar as compared to previously described findings, indicating that PNF is not a limiting factor for survival. Yet, no histological signs of rejection were seen. Again early death occurred in all PVG/c$^-$ rats.

Because of the results obtained in both rat strains, it was hypothesised that the GP liver might be sensitive to handling and ischaemia/reperfusion. After syngeneic GP liver transplantation and GP isolated liver perfusion, GP became increasingly lethargic and eventually died within 2 days.

It is concluded that the PVG/c$^-$ rat is unable to withstand the surgical trauma related to xenogeneic liver transplantation. For both heart and liver grafts, it is deduced that early recipient death occurs because of the surgical trauma and ischaemia/reperfusion injury induced by transplantation of the GP organ. Early recipient/animal death is possibly induced by the release of vaso- and immunoactive substances after GP organ grafting.

Introduction

As the number of patients awaiting transplantation is growing, solutions to deal with this problem fail to keep up. Initiatives, like presumed consent, were unsuccessful, as it increased the amount of donated organs by only a few percent. An infinite pool of organs may become available if animal organs could be used for grafting. The use of species closely related to man, like primates, seems most obvious. However, xenografting of primate organs has various disadvantages, like infection risks and ethical issues. Using the pig as donor animal might be a solution, but rejection is rather vigorous and organs are destroyed by hyperacute rejection (HAR). Preformed natural antibodies and complement initiate this process.

The guinea pig (GP) to rat model is frequently used to study HAR as the rat has preformed natural antibodies against GP. However, lowering of the preformed antibody level still resulted in HAR, indicating that the alternative pathway is the major route of complement activation [1,2]. Indeed, complement depletion by cobra venom factor (CVF) delayed heart graft rejection for several days [3]. GP liver graft survival times were also prolonged, but, surprisingly, no signs of rejection were seen; the recipients died with an apparently functional, non-rejecting graft [4]. Although CVF is a designated non-toxic factor in cobra venom, it might be responsible for this phenomenon. Namely, morphological abnormalities were found in livers from naive rats after CVF-administration (Dr. Scheringa, personal communication). Discordant grafting in a non-CVF-treated, complement-deficient rat, like the PVG/c$^-$ rat, might provide a model in which this issue could be clarified.

We also found that even in untreated GP liver recipients no classical signs of HAR were seen [4]. As in other transplantation models, primary non-function (PNF) might be a possible explanation [5-7]. After a life-supporting transplantation, this could lead to recipient death before the onset of rejection. This hypothesis should include an important role for complement, because CVF-treatment resulted in prolonged survival. A partial auxiliary liver transplantation, in which in time the graft is favoured over the autologous liver, might be supported by the partial remaining recipient liver for the first few days after grafting.

The aim of the present study was, therefore, to investigate possible causes of early recipient death after discordant liver transplantation. The C6-deficient PVG/c$^-$ rat and the partial auxiliary liver transplantation technique were used to study possible CVF-toxicity and PNF in GP liver transplantation. The ensuing results coerced us into performing an isolated liver perfusion experiment.

Materials and methods

Animals

Female Dunkin Hartley GP were used as donors, weighing 150 to 250 grams, and male inbred Brown Norway (BN) and PVG/c$^-$ rats, weighing 200 to 300 grams, as recipients in discordant grafting. For syngeneic liver transplantation and isolated liver perfusion experiments, the used female Dunkin Hartley GP weighed about 275 and 400 grams, respectively. BN rats and GP were purchased from Harlan C.P.B. (Austerlitz, The Netherlands); PVG/c$^-$ rats were obtained from Bantin and Kingman (Edmonds, UK). They were kept under controlled laboratory conditions and received food and tap water ad libitum. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). The "Committee on

Animal Research" of the Erasmus University Rotterdam, The Netherlands, approved the specific protocol.

Liver transplantation procedure

Orthotopic liver transplantation (OLT) was performed according to Kamada et al. [8] with some minor modifications [4]. Atropine was used to pre-treat the donor. The diameters of the Teflon cuffs used for the anastomoses were 2.1 mm and 2.3 mm for the infrahepatic vena cava (IVC) and portal vein (PV), respectively. The bile duct splint had a diameter of 1.1 mm. The donor operation took about 45 minutes, the cuff preparation 15 minutes, and the recipient procedure about 60 minutes. The anhepatic phase did not exceed 25 minutes.

Partial auxiliary liver transplantation (PALT) was performed according to Hess et al. (model D) [9] with some modifications. These modifications included bile duct-bile duct anastomosis and graft IVC anastomosis to the recipient vena cava as proximal as possible without removal of the right kidney. For the IVC, PV, and bile duct anastomoses, a running suture, cuff, and splint were used, respectively. Partial (70%) hepatectomy was conducted on both graft and recipient liver. Recipient bile duct and PV were ligated, whereas the hepatic artery remained intact; no graft arterial flow was established.

Recipient death was taken as endpoint of rejection. The whole transplantation procedure took about the same time as OLT.

Heart transplantation procedure

Heterotopic intra-abdominal heart transplantation (HTx) was performed as described by Ono and Lindsey [10]. The donor operation lasted about 10 minutes and the recipient procedure about 45 minutes. Cessation of heart beating, assessed by palpation, was taken as endpoint of graft survival.

Isolated liver perfusion procedure

Isolated liver perfusion (ILP) was a modification of the technique described by de Brauw et al. [11]. The pyloric branches of the PV and gastroduodenal branch of the common hepatic artery were cannulated, positioning the tips of the cannulas in the hepatic artery and portal vein. To collect hepatic venous outflow a silicon cannula was introduced in the femoral vein and inserted in the vena cava (VC) with the tip positioned at the level of the hepatic veins. Isolation of the hepatic vascular bed was obtained by temporarily ligating the hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the suprahepatic vena cava (SVC) and application of a temporary ligature around the IVC, containing the cannula, cranial to the right adrenal vein. The mesenteric artery was temporarily clamped in order to reduce splanchnic blood pressure. After the procedure, all temporary clamps on the VC, PV, hepatic artery, and mesenteric artery were removed. Gastroduodenal artery, pyloric vein, and femoral vein were ligated. The GP were perfused with oxygenated Haemaccel (Behring Pharma, Amsterdam, The Netherlands) of 38 to 39°C for 10 minutes.

The whole ILP-procedure lasted about 80 minutes.

Complement depletion and determination

Cobra venom (*Naja naja kaouthia*, lot number 57362) was used to deplete complement. It was obtained from ICN Pharmaceuticals B.V. (Zoetermeer, The Netherlands). CVF purification was performed according to Beukelman et al. [12]. One unit of CVF was designated as the amount of CVF causing 50% inhibition of lysis as measured by the classical lytic pathway. The used batch of CVF showed the following characteristics: One single bolus injection of 3000 U CVF iv.

resulted in an immediate decline of complement levels. The levels were below detection limits for at least 4 days, after which the complement activity gradually returns, as measured by the CH50-assay.

Histology

After rejection, necropsy was carried out. The heart graft or upper liver lobe was removed and processed for histology. Formalin fixed tissue was paraffin embedded, cut and stained with haematoxylin-eosine for examination of morphological changes by conventional light microscopy.

Experimental design

PVG/c⁻-experiment: GP heart and liver transplantations were carried out in different PVG/c⁻ recipients. Two groups for both heart and liver graft recipients were studied. Recipients in groups 1 and 3 (n=3 for heart and n=8 for liver, respectively) received no treatment. Intravenous administration of a 1 ml bolus injection of CVF (3000 U/ml) was given to recipients on days -1, 1, 3, etc. (n=4 for heart, group 2; n=4 for liver, group 4).

PALT-experiment: GP PALT was performed in the BN and PVG/c⁻ rat strains. Two groups for both strains were studied. Recipients in groups 1 and 3 (n=5 and n=2, respectively) received no treatment. Groups 2 (n=7) and 4 (n=3) received 3000 U CVF iv. per animal on days -1, 1, 3, etc. After analysing the results, a third experiment was performed.

GP syngeneic OLT/ILP-experiment: For both surgical procedures, four untreated animals were used.

Survival (abdominal palpation or recipient death) was scored in hours/days. At necropsy the upper liver lobe or heart graft was removed and processed for histology. Heart and liver grafts were scored for histological damage.

Statistics

Statistical evaluation was carried out using logarithmic transformation values of the survival data. This was done to reduce the influence of outlying values. Independent T-tests were performed on the PVG/c⁻-experiment data. "One-way" analysis of variance (ANOVA) was performed on the PALT-experiment data. If the ANOVA was significant on a 5% level, post-hoc tests were carried out. Depending on the homogeneity of variances, tested by the Levene test, Games-Howell test or Duncan's multiple comparison test were carried out for possible differences among the means. These tests were corrected for unequal group sizes.

Probability values lower than 0.05 were considered statistically significant. All computing was performed using the statistical software package SPSS for Windows, release 7.5.2.

Results

PVG/c⁻-experiment

Graft survival

Liver grafts showed an inhomogeneous reperfusion. Small parts of the liver lobes remained inadequately perfused. Around 60% of the heart recipients rejected their grafts eventually, whereas all recipients of liver grafts died following reduced respiration and awareness, indicating shock.

Table 1. *Survival times following discordant heart and liver transplantation in PVG/c⁻ rats.*[a]

Group	Surgical procedure	Treatment	Survival	Mean survival
1	HTx	Untreated	(0), (0), 1, 2, 2 days	1,7 days
2	HTx	CVF	(0), (2), 3, 3, 5 days	3,7 days
3	OLT	Untreated	1, 1, 1, 1½, 2, >2, 2½, >3½ hours	1,8 hours
4	OLT	CVF	1, 1, >2, 6 hours	2,5 hours

[a]CVF (groups 2 and 4) was administered on days -1, 1, 3, etc. A significant difference concerning heart graft survival was found between the untreated and CVF-treated groups. Survival times of animals that died with a functioning graft are put between parenthesis.

The survival times are shown in Table 1. For heart graft recipients, a significant difference between survival times of both treatment groups was found (p=0.048). CVF-treatment did not prolong survival times after liver grafting.

Histology

Heart grafts of recipients that died on day 0 revealed no morphological changes. Extravasation, oedema, and few polymorphonuclear granulocytes were seen. CVF-administration showed similar morphological changes as the rejected hearts from untreated recipients. The granulocyte infiltration was more pronounced.

Except from marked reversible hydropic degeneration, no necrosis or infiltration was observed in liver grafts in both treatment groups. Interstitial haemorrhage and capillary and venular congestion were absent.

PALT-experiment

Graft survival

Reperfusion of liver grafts was inadequately in group 1. In the other liver graft groups, this reperfusion problem was reduced but present.

Graft survival times are noted in Table 2. A highly significant difference was found between the group means (p=0.001, s_{res}=0.909). CVF-treatment prolonged survival in the BN rat significantly (p=0.006), but not in the PVG/c⁻ rat. Difference in survival times existed between BN and PVG/c⁻ rats in the CVF-treated situation (p=0.006), but no difference was found in the untreated situation.

Histology

Liver grafts from untreated BN recipients showed marked reversible hydropic degeneration. Neither extravasation nor capillary congestion was seen. Administration of CVF led to severe hydropic degeneration and local necrosis in the grafted livers. No infiltration was seen. The rat that survived for 1.5 hours showed normal architecture Except for slight reversible hydropic degeneration, no abnormalities were found in the

Table 2. *Survival times following discordant partial auxiliary liver transplantation.*[a]

Group	Recipient strain	Treatment	Survival (hours)	Mean survival (hours)
1	BN	Untreated	1, 2, 2¼, 2½, 3	2.2
2	BN	CVF	1¼, 31, >31, >33, >35, >40, 45	30.9
3	PVG/c⁻	Untreated	2, >2	2
4	PVG/c⁻	CVF	1, 2, 5	2.7

[a]Grafts were transplanted in the guinea pig to rat transplantation model. CVF (groups 2 and 4) was administered on days -1, 1, 3, etc. A significant difference between survival times of both treatments in BN rats was found. Between BN and PVG/c⁻ rats, only CVF-treatment differed significantly.

in PVG/c⁻ rat grafted livers. All partial autologous livers demonstrated slight reversible hydropic degeneration. Slides of BN rat lung tissue revealed in 4 out of 7 cases, independent of recipient survival, thickening of the alveolic membranes and haemorrhage.

GP syngeneic OLT/ILP-experiment

The results of the preceding experiments indicated that the use of GP liver in transplantation could have caused early deaths in the discordant transplantation model [4]. To study this syngeneic GP OLT and ILP was performed.

Graft survival

Reperfusion of liver grafts after transplantation was inhomogeneous. None of the GP regained full consciousness and all died within a few hours after OLT. After ILP, small reperfusion lesions were encountered.

Syngeneic GP OLT and GP ILP resulted in survival times as shown by Table 3. One GP survived for 4 hours after ILP without technical complications. Post-mortal examination revealed haemorrhages in the stomach and bowel. The animals that survived more than 1 day became increasingly lethargic around 24 hours after ILP. Eventually, all GP died, following reduced respiration and awareness.

Table 3. *Survival times of guinea pigs following syngeneic OLT or ILP.*

Group	Surgical procedure	Survival (hours)	Mean survival (hours)
1	Syngeneic GP OLT	1, 1½, 2, 2	1.6
2	GP ILP	4†, 14, 36, 48	15.5

†Postmortal examination revealed haemorrhages in stomach and bowel.

Discussion

The reduced susceptibility of liver grafts to antibody-mediated rejection in allografting is an interesting phenomenon studied by various groups [13-15]. In xenotransplantation, the possible occurrence of this phenomenon may be advantageous to the control of rejection. Reduced susceptibility to antibody-mediated rejection has already been suggested in concordant xenogeneic liver grafting [16,17]. Nevertheless, we were unable to investigate this phenomenon in our GP to rat model of discordant liver grafting [4]. The recipients died after several hours to days with an apparently functioning graft. To study the possible causes like CVF-toxicity or PNF, we conducted the current experiments.

To investigate CVF-toxicity, the previously described complement deficient PVG rat (PVG/c$^-$) was used [18]. The PVG/c$^-$ rat has a complement deficiency at C6 and a partial depletion of C2. Antibodies directed against C6 are responsible for the deficiency, but do not explain the partial depletion of C2 [19]. In the GP to PVG/c$^-$ rat heart transplantation model, no influence of the terminal complement complex as the effector is expected. Heart graft survival was prolonged to 1 to 2 days. This is in accordance with results described by others [20]. Nevertheless, 40% of the heart graft recipients died with functioning grafts in the immediate post-operative period. Suh et al. described similar findings in 6 out of 8 cases [21]. Treating PVG/c$^-$ rats with CVF prolonged GP heart survival times even more. As CVF depletes complement downstream of C3, the additive depletion of anaphylatoxins C3a and C5a might be responsible for prolongation of graft survival in the PVG/c$^-$ rat. Although these anaphylatoxins are not the primary mediators of HAR [22], their role in a delayed rejection remains to be elucidated. It remains, however, remarkable that CVF-treatment alone in several rat strains never led to survival times as long as in CVF-treated PVG/c$^-$ rats.

To exclude CVF-toxicity in discordant liver grafting, GP livers were transplanted in PVG/c$^-$ rats. Surprisingly, survival times were similar to survival times found in untreated BN rats [4]. Even administration of CVF to PVG/c$^-$ liver recipient resulted in survival times of only a few hours. No early deaths were encountered in syngeneic PVG/c$^-$ rat OLT [19]. These results, supported by histological findings, therefore, suggest that the PVG/c$^-$ rat is unable to withstand discordant grafting in combination with the surgical trauma related to liver transplantation. This is indicated by early recipient death in both heart and liver transplantation groups in 40% and 100% of the cases, respectively.

Because of the lack of discordant liver graft survival, PNF of the GP liver was also suspected. A discordant PALT was developed to overcome this problem. The remaining recipient liver, after 70% hepatectomy, might bridge the time to recover from PNF.

Moreover, possible endothelial debris, as mentioned by Gridelli et al. [23], and immuno- and vasoactive substances [23] might be cleared from the circulation by the autologous liver for both BN and PVG/c⁻ rat[4]. Transplantation of 30% GP liver in a 70% hepatectomised BN and PVG/c⁻ rat resulted in similar survival times as in normal GP to BN liver transplantation, indicating that PNF is not the reason for early death. Addition of CVF resulted in a mean survival time of 30.9 hours in the BN rat recipients. Compared to the previously published results on CVF-treated GP to BN liver transplantation [4], less variance and little prolongation of survival times is noticed. This is probably due to the capacity of the autologous liver to detoxify the recipient. Again, it is most probable that the PVG/c⁻ rat is unable to cope with the surgical trauma related to xenogeneic liver transplantation.

The results obtained in the present study indicated that CVF-toxicity and PNF might not be the main cause of early recipient death. It was demonstrated that species-specific (non-immunological) characteristics, like anatomy and haemodynamics, confound a possible rejection process in the GP to rat liver transplantation model by reperfusion problems [24]. Moreover, it is known that oedema and bleeding may ensue upon GP organ xenografting, due to the extremely low blood pressure in the GP [25]. Together with the non-immunological problems suggested earlier [4], it was hypothesised that the GP liver might be sensitive to handling and ischaemia/reperfusion. Syngeneic GP to GP OLT was performed to investigate this phenomenon without immunological interference. In accordance with the above-mentioned hypothesis, survival of GP was only a few hours. To eliminate the surgical trauma of OLT, GP ILP was performed. Survival times were significantly longer, but the GP still became lethargic and died after a few hours to days.

This study, therefore, suggests that the PVG/c⁻ rat is unable to withstand the surgical trauma related to xenogeneic liver transplantation. In recipients of heart and especially liver grafts, early death occurs without histological signs of graft rejection. We conclude that this is mediated by surgical trauma and ischaemia/reperfusion injury induced by the transplantation of the GP organ. We hypothesise that release of vasoactive and probably immunoactive substances by the traumatised liver leads to diminished respiratory function and reduced awareness eventually resulting in death.

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CHAPTER FIVE

IgG, but not IgM, mediates hyperacute rejection in hepatic xenografting

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Abstract

We reported earlier that no classical features of hyperacute rejection (HAR) could be found in liver grafts in the guinea pig (GP) to rat model and that recipients died shortly after transplantation, because of non-immunological reasons. Thus, the GP to rat model is not feasible to study the mechanisms of discordant liver xenograft rejection. In the hamster to rat model, long-term survival of a liver graft is possible, but extremely low levels of xenoreactive natural antibodies are present. To mimic a discordant situation with preformed IgM and IgG antibodies, we sensitised rats 1 or 5 weeks before grafting. Specific anti-hamster IgM antibodies were found in recipients sensitised at week -1, but not at week -5. Anti-hamster IgG was present in all recipients, albeit considerably higher in animals sensitised 5 weeks before grafting. In these two models, we examined the mechanism of HAR of liver grafts and compared this with heart xenografts. Control heart and liver grafts rejected in 4 and 7 days after transplantation, respectively. Liver grafts of recipients sensitised at week -5 showed venous congestion and bleeding after reperfusion, indicating HAR. However, this was not observed following sensitisation at week -1. This surprising finding was confirmed by histology. Massive extravasation, oedema and acute liver cell degradation were noticed in grafts subjected to HAR. Liver grafts of recipients sensitised at week -1 showed only minimal histological changes. Heart grafts rejected hyperacutely in both sensitisation models. IgG antibodies could be detected on liver grafts of the group sensitised at week -5, but not the group sensitised at week -1. Minimal IgM depositions were found on liver grafts of animals sensitised 1 week before transplantation. Rejected heart grafts of similar sensitisation groups showed identical antibody depositions, only IgM depositions were massive. Complement depositions were found in all groups. These results indicate that IgG, but not IgM, mediates HAR in hepatic xenografting. Such a predominance of IgG over IgM does not exist for heart grafts.

Introduction

Transplantation of transgenic organs or tissues between widely disparate species as the ultimate solution for donor shortage shows promising results [1]. However, many aspects of rejection are still unknown as evidenced by the muddle of immunosuppressives that are needed to keep pig organs functional. Using small animals, the guinea pig (GP) to rat rodent model provides an easy and fast way to study the basics of discordant transplantation. The recipient bears preformed xenoreactive natural antibodies against the GP, capable of rejecting heart grafts in a hyperacute

manner [2]. These antibodies are mainly of the IgM type [3], but some donor-specific IgG antibodies have been detected [4].

For allogeneic grafting, the relative insensitivity of liver grafts to antibody-mediated rejection has been described [5,6]. In a previous publication, we investigated whether this phenomenon also occurred in the GP to rat liver transplantation model [7]. We demonstrated that a 'rejected' GP liver was not characterised by the classical features of hyperacute rejection (HAR) [7]. However, recipients died within a few days, apparently because of non-immunological reasons. Therefore, no firm conclusions could be drawn regarding the susceptibility of discordant liver grafts toward antibody-mediated rejection [7]. Numerous studies have reported long-term survival of hamster grafts, indicating no interference of non-immunological problems in this model [8-10]. The rat, however, has very low titres of preformed antibodies to hamster. To mimic a discordant situation with preformed IgM or IgG antibodies, we sensitised recipients with donor blood at 1 or 5 weeks before transplantation.

Hence, the aim of the current study was to analyse the mechanism of HAR of liver xenografts in the presence of preformed antibodies and to compare this with the rejection of heart xenografts.

Materials and methods

Animals

Female Syrian hamsters were used as donors and male Brown Norway rats as recipients. All animals were obtained from Harlan C.P.B. (Austerlitz, The Netherlands). They were kept under controlled laboratory conditions and received food and tap water ad libitum. Hamsters weighing over 120 grams and rats weighing between 250 and 300 grams were used. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). The specific protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands.

Liver transplantation procedure

Orthotopic liver transplantation (OLT) was performed according to a previously described method with some donor-related modifications [7,11]. The modifications were: 1. donor pre-treatment with 0.1 mg/kg atropine (Centrafarm Services B.V., Etten-Leur, The Netherlands) and 2. the outside diameters of the Teflon cuffs were 2.1 mm and 1.79 mm for the infrahepatic vena cava and portal vein, respectively. Recipient death was taken as endpoint of rejection.

Heart transplantation procedure

Heterotopic abdominal heart transplantation (HTx) was performed as described by Ono and Lindsey [12]. Cessation of heartbeat as evidenced by abdominal palpation was taken as endpoint of rejection.

Sensitisation

Hamster blood was obtained by orbital punction. One ml of heparinised blood was injected iv. into the penile vein of the recipient 1 or 5 weeks before organ grafting.

Haemagglutination assay

Total antibody and IgG levels were measured using a haemagglutination assay. Plasma samples, taken immediately before grafting, were serially diluted. A suspension of freshly prepared hamster erythrocytes (4%) was added in equal amounts. After 1 hour incubation at 37°C the wells were screened for agglutination. The dilution at which agglutination still occurred was considered the haemagglutination titre. For IgG level measurements, plasma samples were treated with 0.5 mM dithiotreitol for half an hour at 37°C to deplete IgM. Normal BN-serum was taken as negative control. Estimations of IgM titres were calculated by subtracting IgG titres from total antibody titres.

Histology and immunohistochemistry

After rejection of the grafts, necropsy was performed. The heart graft or upper liver lobe was removed and processed for histology and immunohistochemistry. Paraffin sections were stained with haematoxylin-eosine and examined with conventional light microscopy. The slides were examined for extravasation, oedema, vessel damage and infiltration. Changes were noted as compared to naive grafts and scored from - (no changes) to +++ (severe changes)

For antibody, complement depositions and NK-cell infiltration, immunohistochemistry on frozen sections was performed. Macrophages were identified on paraffin sections. FITC-labelled mouse anti-rat antibodies to demonstrate IgM, IgG1, IgG2a and IgG2b were used (1:10; PharMingen, San Diego CA, USA). IgG2c and complement C3 were demonstrated by FITC-conjugated rabbit anti-sheep (1:100; DAKO A/S, Glostrup, Denmark) to sheep anti-rat antibodies (1:500; ANAWA Trading NA, Wangen Zürich, Switzerland). Complement factors C1q and C9 were stained by rabbit anti-rat IgG (1:25, 1:600, respectively, kindly provided by Dr. B.P. Morgan) and secondary FITC-labelled swine anti-rabbit (DAKO A/S). Rat spleen was taken as positive control, whereas liver and heart from naive hamsters were used as negative controls. The slides were analysed by fluorescence microscopy. Location of the depositions was noted and the fluorescence intensity was scored from - (no depositions) to +++ (massive depositions). A 3-step indirect Ni-DAB immunoperoxidase staining was performed on paraffin slides to demonstrate macrophage infiltration. Mouse anti-rat macrophage was used as primary antibody (ED1, 1:800; Serotec Ltd., Oxford, UK). Staining was performed with Ni-DAB substrate after the application of rabbit anti-mouse PO and swine anti-rabbit PO antibodies (1:250; DAKO A/S). Applying the same protocol, NK-cells were demonstrated on frozen sections using mouse anti-rat NK-cell (NKR-P1, 1:400; Endogen, Woburn MA, USA). Conventional light microscopy was used to analyse the infiltration.

Experimental design

Heart and liver transplantations were carried out in different recipients. Sensitisation with 1 ml of hamster blood was performed 1 week (1-week sensitised), for groups 2 and 5, or 5 weeks (5-week sensitised), for groups 3 and 6, before transplantation. Groups 2 and 3 received heart grafts (n=5 for both); groups 5 and 6 obtained liver grafts (n=7 and n=5, respectively). Non-sensitised control groups were included for both heart (group 1, n=7) and liver transplantations (group 4, n=8). On day 0 the recipients obtained a heterotopic hamster heart graft or orthotopic hamster liver graft. Survival, as determined by abdominal palpation or death, was scored in

minutes, hours or days, depending on the treatment. At necropsy the heart graft or upper liver lobe was removed and processed for histology and immunohistochemistry. Heart and liver grafts were semi-quantitatively scored for type and quantity of antibody and histological changes.

Statistics

Statistical evaluation of the survival data was carried out for both heart and liver grafts. In cases of differences in variances as tested by the Levene test, mathematical transformation of the survival data was carried out. This was done to reduce the influence of outlying values. "One-way" analysis of variance (ANOVA) was performed on these data. If the ANOVA was significant on a 5% level, the Duncan's multiple comparison test or Games-Howell test was carried out for possible differences among the means. The tests were corrected for the fact that the comparisons were not statistically independent and for unequal group sizes.

Probability values lower than 0.05 were considered statistically significant. Survival of transplants with clear evidence of no rejection (liver enzymes, histology) was discarded from statistical evaluation. All computing was performed using the statistical software package SPSS for Windows, release 7.5.2.

Results

Histology

Specimens of the transplanted organs were obtained for histological examination after sacrificing the animals at the moment of rejection or death. Heart grafts rejected by non-sensitised rats showed vessel destruction, extravasation, oedema and polymorphonuclear cell infiltrate, suggesting an antibody-mediated rejection (see Figure 1A). Presence of mononuclear cells was also noted. Groups 2 and 3 showed more severe extravasation of erythrocytes, fibrosis and oedema (see Figure 1B, C). The overall architecture, including most vessels, was intact. In some cases, polymorphonuclear granulocytes were found.

Control liver grafts demonstrated polymorphonuclear infiltrate, destroyed vascular morphology with damaged endothelial cell layers and oedema (see Figure 1D). Compared to heart grafts, a more predominant mononuclear cell infiltrate consisting of lymphocytes, lymphoblasts and monocytes and macrophages was also seen. This indicates a mixture of cellular as well as antibody-mediated rejection. The infiltrate was situated around the portal areas.

Liver grafts of 1-week sensitised recipients showed extravasation, oedema, signs of fibrosis and vascular congestion but little vessel damage (see Figure 1E). Focally, mononuclear granulocytes were found. Specimens of liver grafts in group 6 revealed extensive tissue damage, extravasation and acute liver cell damage (see Figure 1F). Semi-quantitative histology scores are listed in Table 1.

Table 1. *Histological changes in heart and liver grafts rejected by sensitised recipients.*

Group	Treatment	Extravasation	Oedema	Vessel damage	Infiltration
1	HTx, non-sensitised	+	++	++	+++
2	HTx, 1-week sensitised	++	++	+	±
3	HTx, 5-week sensitised	++	++	+	±
4	OLT, non-sensitised	+	++	++	+++
5	OLT, 1-week sensitised	+	+	+	±
6	OLT, 5 week-sensitised	+++	+	+++	+

Scores varied from - (no changes) to +++ (severe changes) compared to naive heart/liver.

Immunohistochemistry

Frozen sections of heart and liver grafts were stained for IgM, IgG subtypes, complement C1q, C3 and C9 and NK-cell infiltrates. Paraffin sections were used to stain for macrophages. All control stainings were positive on untreated rat spleen, whereas untreated hamster liver and heart sections were negative.

Control hearts showed major IgM depositions (see Figure 2A). Some IgG2a type antibodies and macrophages were noticed. Liver grafts from untreated rat recipients showed minor antibody depositions on cellular infiltrates or portal fields, mainly being IgM (see Figure 2C). Macrophages, but not NK-cells were deposited throughout the tissue.

Heart grafts from 1-week sensitised recipients demonstrated massive antibody depositions, whereas liver grafts demonstrated minor depositions (see Figure 2B, D and Figure 3A, B). In group 3 and 6, moderate IgG1 depositions could be demonstrated. In addition, depositions of IgG2a and IgG2b were detected on myocytes. In liver grafts, depositions of IgG2b were found. Semi-quantitative antibody deposition scores are listed in Table 2.

Table 2. *Antibody depositions on heart and liver grafts rejected by sensitised recipients.*

Groups	Treatment	IgM	IgG1	IgG2a	IgG2b	IgG2c
1	HTx, non-sensitised	+++	-	+	-	-
2	HTx, 1-week sensitised	+++	-	-	-	-
3	HTx, 5-week sensitised	-	++	+	+	-
4	OLT, non-sensitised	+	±	±	±	±
5	OLT, 1-week sensitised	±	-	-	-	-
6	OLT, 5-week sensitised	-	++	±	+	-

Scores varied from - (no depositions) to +++ (massive depositions).

Liver grafts from untreated rat recipients showed some C1q depositions, but no C3 and C9, whereas all measured complement components could be detected on heart grafts. Sensitising rats 1 week before transplantation resulted in moderate C3 depositions in both heart and liver grafts. In addition, positive Kupffer-cells were found. C1q could only be detected in heart grafts whereas little C9 was found in liver grafts. In 5-week sensitised recipients, massive C3 and moderate C9 depositions were demonstrated for heart grafts. Moderate C3 depositions and to a lesser extend C1q could be shown for liver grafts in group 6.

Graft survival

Heart and liver grafts showed a homogeneous reperfusion after releasing the clamps. After a few minutes, the heart grafts turned dark red. Liver grafts in group 6, but not group 5, were purple and showed swelling 2 minutes after reperfusion. Most of the recipients of liver grafts died showing the clinical signs of shock.

Graft survival times are shown in Table 3. A highly significant difference was demonstrated between the group means in the heart-transplant groups ($p=0.000$, $s_{res}=35.730$) and between the group means in the liver-transplant groups ($p=0.000$, $s_{res}=0.567$). Non-sensitised heart graft recipients showed prolonged survival compared with both other heart graft recipients ($p<0.001$, both). No difference was found between 1-week sensitised and 5-week sensitised animals. Survival times of the non-sensitised group receiving liver grafts were significantly longer than those of animals in groups 5 and 6 ($p=0.000$, both). Survival times in groups 5 and 6 were not significantly different but a trend was noticed ($p=0.081$).

Table 3. *Survival times following 'pseudo-discordant' heart and liver transplantation.*[a]

Group	Surgery	Treatment	Survival times		Median survival
1	HTx	non-sensitised	3, 4, 4, 4, 4, 4, 4	days	4 days
2	HTx	1-week sensitised	5, 6, 7, 120, 165	minutes	7 min.
3	HTx	5-week sensitised	3, 3, 3, 9, 10	minutes	3 min.
4	OLT	non-sensitised	(3), (4), 6, 6, 7, 7, 8, 8	days	7 days
5	OLT	1-week sensitised	1½, 1½, 2, 2½, 3, 21, 25	hours	2½ hours
6	OLT	5-week sensitised	1, 1, 1¼, 1¼, 1½	hours	1¼ hours

[a]Hamster heart or liver was grafted in BN rat following a hamster blood sensitisation at 1 week (group 2 and 5) or 5 weeks (group 3 and 6) before transplantation. In control groups 1 and 4, hamster heart or liver was grafted into naive BN rat. Both control groups were statistically different from their sensitisation groups. Survival times of animals that died with a functioning graft are put between parenthesis.

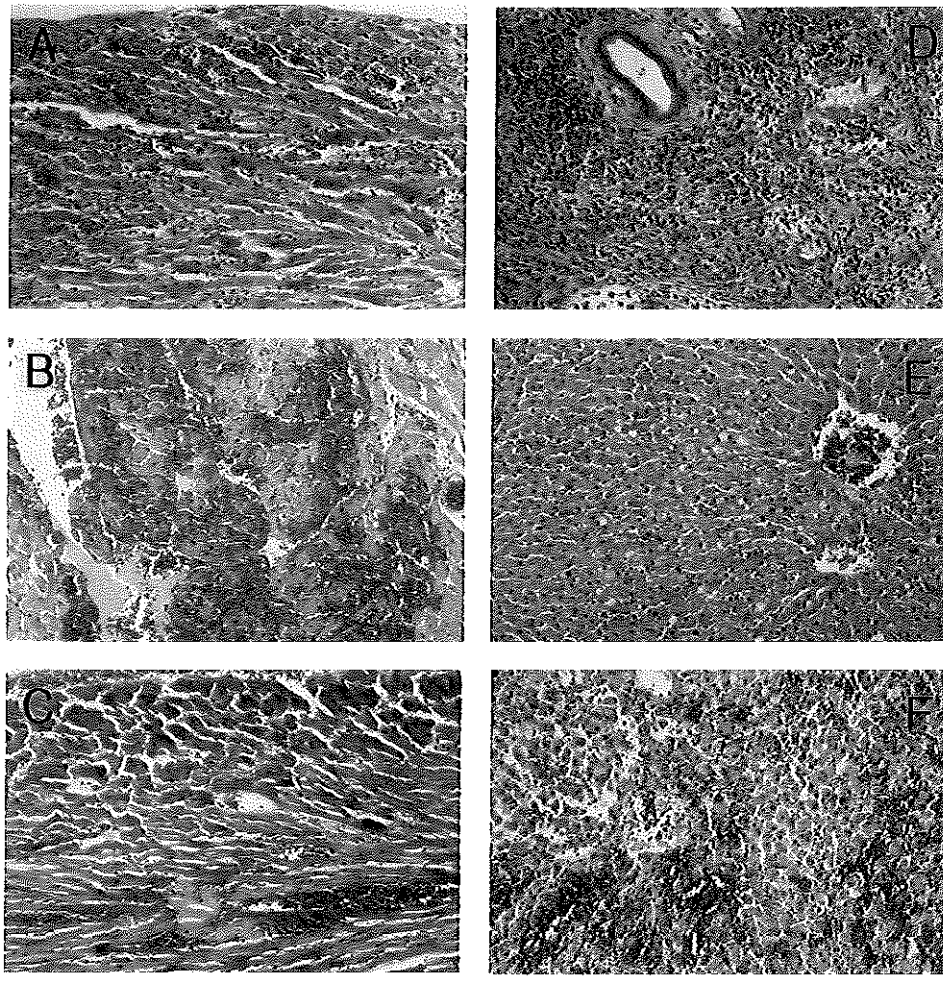

Figure 1. *Haematoxylin-eosine stained heart and liver graft sections.*
Rejected hamster heart at day 4 (A). Oedema and polymorphonuclear cell (PMN) infiltrate are nicely depicted. Hyperacutely rejected hamster hearts after 1 week (B) and 5 weeks (C) of sensitisation. Clear evidence of oedema and extravasation of erythrocytes is demonstrated. Rejected hamster liver graft at day 8 (D). Severe infiltration with PMN and mononuclear cells is shown. Oedema is also noticed. Hyperacutely 'rejected' hamster livers after 1 week (E) and 5 weeks (F) of sensitisation. The absence and presence of haemorrhage is shown 1 week and 5 weeks after sensitisation, respectively. Extreme differences were also noticed for vessel damage. (amplifications: ×40)

IgM and IgG agglutination titres

Haemagglutination tests revealed the presence of specific anti-hamster IgM antibodies in 1-week sensitised animals on day 0. The IgM titre was negligible in recipients sensitised 5 weeks before grafting. Anti-hamster IgG was present in all recipients, albeit considerably higher in recipients sensitised at week -5. Normal BN-serum revealed

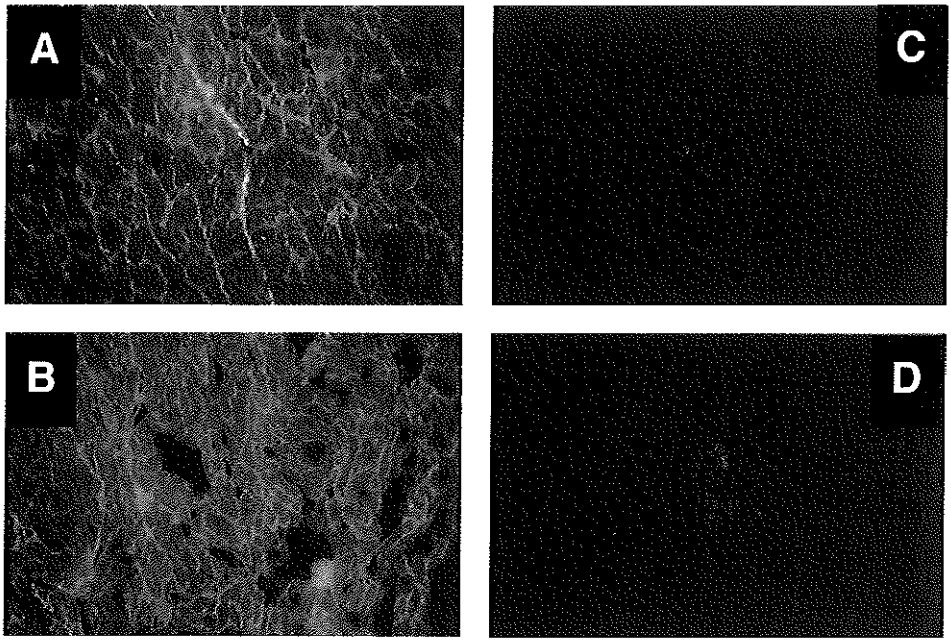

Figure 2. *FITC-labelled IgM stained heart and liver cryostat sections.*
Rejected hamster heart and liver grafts in non-sensitised (A, heart; C, liver) and 1 week sensitised (B, heart; D, liver) recipients. Severe IgM depositions were noticed only on heart grafts of non-sensitised and 1 week sensitised recipients, whereas only marginal IgM depositions on cellular infiltrates are shown on liver grafts in non-sensitised recipients. (amplifications: ×40)

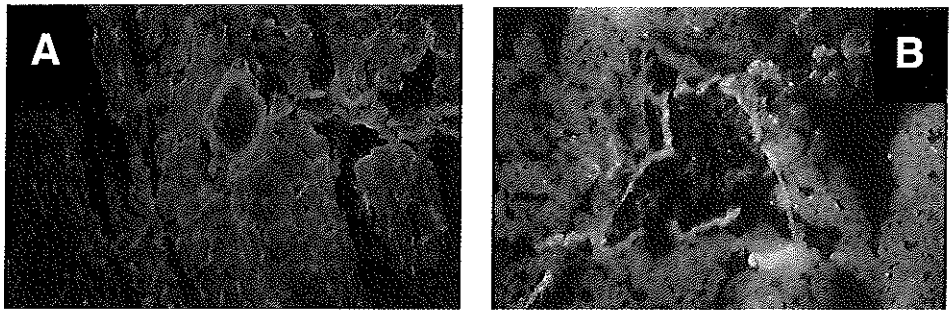

Figure 3. *FITC-labelled IgG1 stained heart and liver cryostat sections.*
Rejected hamster heart and liver grafts in 5 weeks sensitised (A, heart; B, liver) recipients. Moderate IgG1 depositions are demonstrated on both heart and liver grafts. (amplifications: ×40)

agglutinating titres of ¼ or lower. No statistically significant correlation was found between pre-operative antibody titre and survival or histological changes in either sensitisation group.

Discussion

The immuno-privileged position of liver grafts to antibody-mediated rejection has been a research subject for many years. Preformed antibodies in the circulation of patients before transplantation have been identified as the cause of HAR of allografts [13]. However, hyperacute antibody-mediated rejection is not necessarily observed [5,14]. Liver allografts seem to be less susceptible to antibody-mediated rejection. The phenomenon of reduced susceptibility has been demonstrated in previous studies, concerning experimental liver allotransplantation in (sensitised) recipients [6,15]. In discordant liver grafting, we and others were unable to resolve this question, because the recipients died from non-immunological problems [7,16]. For that reason, we performed the current experiments.

Similarly, as described by others, we found that non-sensitised controls rejected hearts significantly earlier than liver grafts [10,17]. Histologically, control heart grafts showed a prominent vascular rejection process, whereas rejected liver grafts (group 4) revealed a more predominant cellular rejection with mononuclear cell infiltrates. Moreover, antibody depositions of the IgM type were more pronounced on heart grafts than liver grafts. These findings indicate that in the untreated concordant hamster to rat combination the liver might be less affected by IgM.

It can be argued that liver grafts are less immunogenic compared to heart grafts, resulting in a diminished and sluggish antibody response resulting in less damage. However, this is not very plausible, because anti-donor antibody levels peak around day five to seven for both liver and heart transplantation [18-20]. Moreover, Murase et al. reported that cytotoxic antibody titres after xenogeneic liver grafting are 10 times higher than after heart grafting [21]. Infusion of hamster hepatocytes or non-parenchymal liver cells was able to induce high cytotoxic antibody titres within 1 week and splenic response to liver grafts is even higher compared to cardiac grafts [22,23]. This indicates that liver grafts may be at least as immunogenic as heart grafts.

Another plausible explanation is that the liver graft protects itself, because the source of complement and the target organ are the same, leading to prolonged survival [24]. This is in accordance with the time required to transform the proteins to donor type profile [7,25]. Nevertheless, residual recipient complement components and neo-synthesis, by macrophages, monocytes, and fibroblasts, are still able to cause lysis [24].

In normal BN-serum, haemagglutinating antibodies were almost non-detectable. In the discordant situation, grafted organs encounter high titres of preformed antibodies. Immunogenicity is, therefore, of less relevance than the affinity of antibodies for the different tissues. To mimic the preformed antibody situation, we sensitised rat recipients with hamster antigen in order to evoke an antibody response. This sensitisation, 1 or

5 weeks before transplantation, resulted in graft survival of minutes to hours for both liver and heart grafts. The differences found between liver and heart graft survival in the sensitised situation may be explained by the difference in survival readout for the grafts. It is known that rats can live for several hours after total hepatectomy with just a portal caval shunt [7].

At necropsy, massive antibody depositions could be detected in heart grafts from 1-week sensitised recipients. Extravasation of erythrocytes, oedema, fibrosis and vascular congestion, suggesting complement-mediated endothelial damage, indicate an ongoing HAR. Liver grafts from recipients sensitised 1 week before transplantation, however, showed an overall intact morphology and only marginal signs of HAR. This corresponds to their phenotypic appearance after grafting. Yet, classical signs of HAR were noted in liver grafts from 5-week sensitised animals: acute liver cell degradation, extensive tissue damage and extravasation of erythrocytes. Purple recolouration and swelling were noticed after reperfusion. Immunohistochemistry showed IgG depositions, whereas no graft depositions could be found in rats sensitised at week -1.

Circulating IgM, present 1 week after sensitisation, is likely to be deposited on heart as well as on liver grafts. However, the results indicate that liver grafts, in contrast to heart grafts, seem to be less affected by IgM as seen by immunohistochemisty and complement depositions. One possible reason is that the liver is releasing blocking agents, preventing antibody deposition, which has been hypothesised by Kamada et al. [26]. Another explanation is that graft size difference gives rise to difference in deposition density and, therefore, rejection. A more likely possibility is that Kupffer-cells absorb large amounts of lymphocytotoxic antibodies [27,28]. Moreover, Crafa et al. reported that Kupffer-cell activation led to significant reduced circulation of anti-donor antibodies and a more intense IgM-uptake after discordant liver grafting as demonstrated by immunohistochemistry [29]. In the present study, however, liver grafts showed minor IgM depositions. Other authors have reported similar findings. Tuso et al. demonstrated that xenogeneic extracorporeal liver perfusion resulted in minimal immunohistochemical evidence of binding of human xenoantibodies [30]. Nevertheless, reduced antibody binding to other organs was found after xenogeneic liver perfusion [30]. Even prolonged heart xenograft survival has been reported after preceding xenogeneic liver transplantation [31]. This indicates that liver grafts, unlike heart grafts, are less susceptible to antibody-mediated damage, probably because of absorption or non-binding of antibodies, possibly IgM.

Five weeks after sensitisation, mainly IgG type antibodies prevail. Histology of rejected livers showed a severe HAR with IgG and complement deposition, which were not detected in the grafts after recipient sensitisation at week -1. It seems, therefore, that IgG, specifically some IgG subtypes, may be responsible for the rejection process in

liver grafts. In addition, several authors found that in experimental allogeneic liver transplantation after recipient sensitisation graft failure was dependent on antibody class, being IgG [32,33]. Moreover, IgG, in contrast to IgM, seems to be the most dangerous in clinical allotransplantation across positive crossmatches [34,35]. The minor damage seen in liver grafts of 1-week sensitised recipients might be caused by the already formed, relative low titres of IgG.

This could also explain why in discordant grafting no signs of HAR were seen, since most XNA are of the IgM type [3,4,7,36].

In conclusion, this study suggests a more dominant role for IgG over IgM antibodies in the rejection of liver xenografts. Such predominance does not exist for heart grafts.

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CHAPTER SIX

Comparative study of liver and heart graft survival in complement-depleted and T-cell suppressed rats

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Abstract

In this study the antibody-mediated cellular rejection of hamster to rat liver xenografts was investigated and compared to the rejection of heart xenografts. Antibody-dependent cellular cytotoxicity (ADCC) was allowed to become apparent by eliminating other potential xenograft rejection processes. The most noticeable processes, complement-mediated and T-cell mediated rejection, were abolished by combined complement depletion and cyclosporine A (CsA) treatment.

Heart grafts in the untreated and CsA-treated (25 mg/kg) recipients survived 3 to 5 days, whereas liver grafts survived 6 to 8 days (p=0.000). After complement depletion by cobra venom factor (CVF), mean survival times in heart and liver grafts were prolonged to 6 and 8.2 days, respectively. Combining T-cell immunosuppression with CsA and decomplementation by CVF did not result in additional prolongation. As histology indicated a combined antibody-mediated and cellular rejection with mild IgM depositions and macrophage infiltration, ADCC was most probably responsible for rejection.

In a second experiment, an initial 50 mg/kg CsA and CVF were used. In this design, 50% of the heart grafts survived long-term, whereas all liver recipients died with functioning grafts within 4 days after OLT. Severe emaciation (>25%) following the surgical trauma was noticed.

In summary, in concordant liver graft rejection, as in heart graft rejection, complement plays an important role. In the absence of T-cell and complement-mediated processes, ADCC is responsible for rejection as indicated by histology.

Introduction

Since the beginning of the 80s, the use of widely disparate species as organ or tissue donors has been considered as the ultimate solution for donor shortage. At present, the latest findings in transplantation of organs from transgenic animals are promising [1]. However, a multitude of immunosuppression is needed to keep such organs functional, which implies that many immunological aspects of rejection mechanisms are still unknown. Providing an easy and fast model, the guinea pig to rat rodent model has been used frequently to study the basics of discordant grafting. One of the basic findings was that preformed xenoreactive natural antibodies, but mainly complement activation via the alternative pathway, are capable of rejecting guinea pig heart grafts in a hyperacute manner [2,3].

Reduced susceptibility of liver allografts to antibody-mediated rejection has been a research subject for many years [4-6]. In a previous publication, the occurrence of this

phenomenon was investigated in the discordant guinea pig to rat liver transplantation model [7]. We found that, although recipients died within a few days, histologically, no classical features of hyperacute rejection were present. Survival was hindered by non-immunological problems, so that no firm conclusions could be drawn regarding susceptibility toward antibody-mediated rejection [7]. In the hamster to rat model, long-term survival has been reported [8], indicating no interference of non-immunological problems [9,10]. After mimicking discordancy in this model by pre-immunisation, it was found that IgG plays a more dominant role in rejection of liver xenografts than IgM and that this predominance does not exist for heart grafts [11].

Apart from hyperacute rejection, it has been demonstrated that cell-mediated rejection of xenografts may also be antibody dependent [12]. Several authors have already indicated that antibody dependent cell-mediated cytotoxicity (ADCC) may arise after complement depletion and inhibition of T-cells and that this could be responsible for xenograft rejection [13,14].

As the liver is an immuno-privileged organ in allotransplantation, the aim of the present study was to investigate whether liver xenografts, compared to heart grafts, would also be less susceptible to rejection in a situation where antibody-mediated cellular rejection is allowed to become apparent.

Material and methods

Animals

For the transplantation procedure, female Syrian hamsters were used as donors. The weight of the animals was 130 grams on average. Male inbred Brown Norway rats were used as recipients of liver and heart grafts and weighed between 250 and 300 grams. All animals were obtained from Harlan C.P.B. (Austerlitz, The Netherlands). They were kept under controlled laboratory conditions and received food and tap water ad libitum. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). The specific protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands.

Procedure liver transplantation

Orthotopic liver transplantation (OLT) was performed according to Kamada et al. with some minor modifications [7,15]. Atropine was used to pre-treat the donor. The diameters of the Teflon cuffs used for the anastomoses were 2.1 mm and 1.79 mm for the infrahepatic vena cava and portal vein, respectively. The bile duct splint had a diameter of 0.63 mm. The donor operation took about 45 minutes, the cuff preparation 15 minutes, and the recipient procedure about 60 minutes. The anhepatic phase did not exceed 25 minutes. Recipient death was taken as endpoint of rejection.

Procedure heart transplantation

Heterotopic intraabdominal heart transplantation (HTx) was performed as described by Ono and Lindsey [16]. The donor operation lasted about 10 minutes and the recipient procedure about 45 minutes. Cessation of heart beating, assessed by palpation, was taken as endpoint of graft survival.

Liver enzyme monitoring

Liver enzymes were monitored just before and after transplantation. As parameters of cellular damage, serum alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined. Alkaline phosphatase (AP) and γ-glutamyl transpeptidase (γGT) were used as parameters indicating bile duct obstruction. Total bilirubin (tBIL) and cholinesterase (CHE) were measured as parameters for liver function.

Complement depletion and determination

Cobra venom (*Naja naja kaouthia*, lot number 57362, ICN Pharmaceuticals B.V. Zoetermeer, The Netherlands) was used to deplete complement. Cobra venom factor (CVF) purification was performed according to Beukelman et al. [17]. One unit of CVF was designated as the amount of CVF causing 50% inhibition of lysis as measured by the classical lytic pathway. The used batch of CVF showed the following characteristics: one single bolus injection of 3000 U CVF resulted in an immediate decline of complement levels. The levels were below detection limits for at least 4 days, after which the complement activity gradually returns, as measured by the CH50-assay. In recipient sera, complement activity of the classical and alternative pathways, the CH50- and AH50-assay, respectively, were measured [18]. Complement depletion after CVF-treatment was complete in all cases.

Histology and immunohistochemistry

After rejection, necropsy was done. The heart graft or upper liver lobe was removed and processed for histology and immunohistochemistry. Formalin fixed tissue was paraffin embedded, cut and stained with haematoxylin-eosine for examination of morphological changes by conventional light microscopy.

For antibody, complement depositions and NK-cell infiltration, immunohistochemistry on frozen sections was performed. Macrophages were identified on paraffin sections. FITC-conjugated mouse anti-rat antibodies were used to demonstrate IgM, IgG1, IgG2a and IgG2b (1:10; PharMingen, San Diego CA, USA). IgG2c and complement C3 were demonstrated by sheep anti-rat antibodies (1:500; ANAWA Trading NA, Wangen Zürich, Switzerland) and stained with FITC-labelled rabbit anti-sheep (1:100; DAKO A/S, Glostrup, Denmark). Complement factors C1q (1:25) and C9 (1:600) were stained by FITC swine anti-rabbit (1:100; DAKO A/S) to rabbit anti-rat IgG (a kind gift of Dr. B.P. Morgan). The slides were analysed by fluorescence microscopy. Location of the depositions was noted and the fluorescence intensity was scored from - (no depositions) to +++ (massive depositions).

A 3-step indirect Ni-DAB immunoperoxidase staining was performed on paraffin sections to demonstrate macrophage infiltration. Mouse anti-rat macrophage (ED1, 1:800; Serotec Ltd., Oxford, UK) was used as primary antibody. Staining was done with Ni-DAB substrate after the application of rabbit anti-mouse PO and swine anti-rabbit PO antibodies (1:250; DAKO A/S). Applying the same protocol, NK-cells were demonstrated on frozen sections using mouse anti-rat NK-cell antibody (NKR-P1, 1:400; Endogen, Woburn MA, USA). Conventional light microscopy was used to analyse the infiltration.

Liver and heart from untreated hamsters were taken as negative controls for anti-rat marker antibodies. As positive control rat spleen was used. All stainings were positive on untreated rat spleen, whereas untreated hamster liver and heart sections were negative.

Experimental design

Heart and liver transplantations were carried out in different recipients. Four groups for both heart and liver graft recipients were studied. Recipients in group 1 and 5 (n=5 for heart and n=8 for liver, respectively) received no treatment. Intramuscular administration of 25 mg/kg cyclosporine A (CsA) on days -1, 1, 3, etc. was added to achieve inhibition of T-cell inhibition (n=7 for heart, group 2; n=7 for liver, group 6). In groups 3 and 7 (n=6 for both), recipients received a 1 ml bolus injection of CVF (3000 U/animal) iv. on days -1, 1, 3, etc. Rats in groups 4 and 8 (n=4 for heart, n=7 for liver, respectively) received the combination of CsA and CVF.

In the second experiment, a combination of 50 mg/kg CsA and 3000 U/animal CVF, administration on days -1, 1, 3, etc. for both substances, was used (n=4 for heart, group 1; n=4 for liver, group 2). On day 14, 28 and 42, CsA administration was lowered to 25, 12.5 and 5 mg/kg, respectively.

Survival time (abdominal palpation or recipient death) was scored in days. Heart and liver grafts were scored for type and quantity of antibody and examined for morphological changes.

Statistics

Statistical evaluation was done using logarithmic transformation values of the survival data. This was done to reduce the influence of outlying values. "One-way' analysis of variance (ANOVA) was performed on these data. If the ANOVA was significant on a 5% level, post-hoc tests were carried out. Depending on the homogeneity of variances, tested by the Levene test, Games-Howell test or Duncan's multiple comparison test were carried out for possible differences among the means. These tests were corrected for unequal group sizes.

The T-test was used to compare the logarithmic transformation values from survival data of heart and liver grafts within one treatment.

Probability values lower than 0.05 were considered statistically significant. Survival of transplants with clear evidence of no rejection (liver enzymes, histology) was discarded from statistical evaluation. All computing was done using the statistical software package SPSS for Windows, release 7.5.2.

Results

Graft survival

Liver and heart grafts showed a homogeneous reperfusion after releasing the clamps. Eventually, most heart graft and liver graft recipients rejected their transplant. Animals that died with functional grafts were discarded from evaluation.

Graft survival times are shown in Table 1. A highly significant difference was found between the group means in the heart transplantation experiments ($p=0.000$, $s_{res}=0.152$). CsA-treatment did not prolong the survival. The administration of CVF to recipients after HTx significantly prolonged the survival compared to untreated controls ($p=0.001$) and CsA-treated rats ($p=0.007$). The combination of CsA- and CVF-therapy

Table 1. *Survival times following concordant heart and liver transplantation.*[a]

Group	Surgical procedure	Treatment	Survival times (days)	Mean survival (days)
1	HTx	Untreated	3, 3, 3, 3, 4	3.2
2	HTx	CsA25	3, 3, 3, 4, 4, 5, 5	3.9
3	HTx	CVF	6, 6, 6, 6, 6, 6	6
4	HTx	CsA25 + CVF	5, 5, 5, 6	5.3
5	OLT	Untreated	(1), 6, 6, 7, 7, 7, 7, 8	6.9
6	OLT	CsA25	(2), 6, 6, 6, 8, 8, 8	7.0
7	OLT	CVF	(5), 6, 7, 9, 9, 10	8.2
8	OLT	CsA25 + CVF	(3), 7, 8, 8, 9, 10, 11	8.8

[a]Grafts were transplanted in the hamster to rat transplantation model. Cyclosporine A (CsA, 25 mg/kg) (groups 2 and 6) and cobra venom factor (CVF, 3000 U/animal) (groups 3 and 7) were administered on days -1, 1, 3, etc. Groups 4 and 8 received the combination treatment. Significant differences concerning heart graft survival times were found between the non-CVF-treated groups and the CVF-treated groups. For liver grafts, only group 5 and 8 differ significantly. For all treatments, heart and liver graft survival differs significantly. Survival times of animals that died with a functioning graft are put between parenthesis.

(group 4) did result in a significant extended survival compared to untreated controls ($p=0.001$) and the CsA-group ($p=0.04$).

A significant difference was found between group means after liver grafting ($p=0.034$, $s_{res}=0.158$). CsA-treatment combined with CVF resulted in significantly prolonged survival times compared to untreated controls ($p<0.05$). No significant differences were found between other schedules.

Comparison of the heart and liver graft survival data with the same treatment schedules resulted in significant differences in all groups ($p=0.000$ between groups 1 and 5, $p=0.000$ between groups 2 and 6, $p=0.035$ between groups 3 and 7 and $p=0.001$ between groups 4 and 8).

Table 2. *Survival times following concordant heart and liver transplantation.*[a]

Group	Surgical procedure	Treatment	Survival times (days)	Mean survival (days)
1	HTx	CsA50 + CVF	(4), (7), 67, 70	68.5
2	OLT	CsA50 + CVF	(1), (2), (4), (4)	-

[a]Grafts were transplanted in the hamster to rat transplantation model. Cyclosporine A (CsA, 50 mg/kg) and cobra venom factor (CVF, 3000 U/animal) were administered on days -1, 1, 3, etc. Survival times of animals that died with a functioning graft are put between parenthesis.

In the second experiment, 50% of the heart graft recipients survived long-term (Table 2). Two xenografts survived for more than 25 days on a low CsA-treatment, being 5 mg/kg on alternating days. The other two animals died with functioning grafts. Over 25% weight loss was noticed in all animals, which reversed after lowering the CsA-dosage. None of the liver graft recipients survived for more than 4 days.

Histology

Specimens of the transplanted organs for histological examination were obtained after sacrificing or death of the animals. Control heart grafts showed extravasation, oedema and vascular destruction, primarily indicating an antibody-mediated rejection. No other histological changes were seen after CsA-treatment, compared to the untreated group. Heart grafts sections demonstrated considerable more cellular infiltration after CVF-treatment than control hearts. Especially polymorphonuclear granulocytes and monocytes were seen, indicating ADCC. Group 4 heart grafts also showed similar signs of rejection as seen in group 3.

Control liver grafts showed a monocyte, lymphocyte and lymphoblast infiltrate and to a lesser extent polymorphonuclear granulocytes, suggesting a combination of a vascular and cellular rejection. CsA-treatment in liver grafting resulted in a reduction in monocyte infiltration, compared to the untreated recipients. Extravasation of erythrocytes and infiltration of granulocytes was more pronounced. Slides of liver grafts in group 7 revealed mild to extensive monocyte infiltration, depending on the survival times. Some grafts displayed vessel damage, extravasation and a granulocyte infiltrate but this was less notable when compared to groups 5 and 6. Histological examination of group 8 revealed similar but more prominent findings as seen in the CVF-treated liver recipients. Extensive cellular infiltration by monocytes, lymphocytes and some plasma cells, vessel and parenchymal damage and extravasation of erythrocytes was noticed, indicating a combined antibody-mediated and cellular rejection.

The occurrence of the pathological findings was more or less dependent on survival times.

In the second experiment, slides of heart grafts revealed extensive polymorphonuclear granulocyte infiltration and only small numbers of monocytes, indicating a dominant vascular rejection. Marginal artheriosclerotic lesions were present. No histological changes were noted in liver grafts in group 2.

Immunohistochemistry

Heart grafts from untreated and CsA-treated recipients showed depositions of IgM and IgG2a along myocytes and vessels. After CVF-treatment and combined CsA- and CVF-treatment, heart grafts demonstrated severe depositions of IgM and mild IgG.

Table 3. *Median antibody depositions in rejected heart and liver grafts.*[a]

Groups	Treatment	IgM	IgG1	IgG2a	IgG2b	IgG2c
1	HTx, none	+++	-	+	-	-
2	HTx, CsA25	+++	-	+	-	-
3	HTx, CVF	+++	-	++	-	-
4	HTx, CsA25/CVF	+++	-	++	-	-
5	OLT, none	+	±	±	±	±
6	OLT, CsA25	+	-	±	±	±
7	OLT, CVF	+	+	±	-	±
8	OLT, CsA25/CVF	++	+	+	±	+

[a] Scores varied from - (no depositions) to +++ (massive depositions).

In liver grafts from untreated and CsA-treated rat recipients, marginal antibody depositions, being mainly IgM, were detected on cellular infiltrates and in portal fields. Macrophages, but not NK-cells, were deposited all throughout the tissue. Treatment with CVF showed more IgG depositions, especially IgG1, compared to untreated liver grafts recipients and depending on survival. When additionally treated with CsA, mild to massive IgM depositions on hepatocytes and Kupffer-cells were noticed. Also mild IgG subtype depositions on cellular infiltrates and parenchymal cells was present. Antibody depositions were related to survival times. Semi-quantitative antibody deposition scores are listed in Table 3.

Macrophages were detected in both heart and liver grafts, whereas only heart grafts showed marginal amounts of NK-cells. No correlation was found between cellular influx and survival times.

Heart grafts of untreated and CsA-treated recipients showed mild complement C1q, C3 and C9 depositions along vessels and myocytes. In the CVF-treated and combination treatment group, marginal to mild depositions of C1q were found. Marginal C1q depositions were found on parenchymal cells in portal fields in groups 5 and 6. In groups 7 and 8, C1q depositions were more intense and also deposited along vessel walls. No complement C3 and C9 could be found in liver grafts of all groups.

Discussion

The relative resistance of the liver to antibody-mediated rejection compared to other organs has been a research subject for many years. Circulating preformed antibodies have been identified as the cause of hyperacute rejection in allografting [19]. However, in liver grafts, hyperacute antibody-mediated rejection is not necessarily observed [4,20]. Reduced susceptibility of liver grafts to antibody-mediated rejection has also been suggested in xenografting [21,22]. In a previous publication on discordant liver

grafting, we were unable to investigate this phenomenon: the animals died prematurely from non-immunological reasons [7]. By sensitisation of rat recipients in a concordant model, we imitated discordancy and found a predominance of donor-specific IgG over IgM in rejecting liver grafts [11]. No such predominance could be detected for heart grafts. To study the assumed immuno-privileged position of the liver for antibody dependent cellular cytotoxicity, we conducted the current experiments.

As described by others, we found that untreated recipients rejected hearts significantly earlier than liver grafts [10,23]. The fact that antibody type switching occurs a few days after transplantation and that liver grafts possess a reduced susceptibility to IgM might explain these differences [11]. Histologically, control heart grafts showed a prominent vascular rejection process, whereas rejected liver grafts revealed a more predominant cellular rejection with mononuclear cell infiltrates, although also polymorphonuclear granulocytes were observed. In accordance with our earlier findings [11], antibody depositions of the IgM type were found to be more pronounced on heart grafts than liver grafts.

The influence of T-cell mediated processes on both heart and liver graft rejection is probably marginal, as shown by the absence of survival prolongation by CsA-treatment. These results are supported by other authors [24,25]. Additionally, it was found that also T-cell depleting substances, like CD4-, CD8 and TCR-antibodies, were unable to prolong heart graft survival [26,27]. On the other hand, the effective T-cell suppressant FK506, ineffective in prolonging hamster heart survival, resulted in long-term survival in 3 out of 10 liver transplants [10].

Depletion of complement resulted in a significant 2-days prolongation for heart grafts and a 1-day prolongation for liver grafts. Survival times of heart grafts are in accordance with the results found by others [14,26]. Prolongation of liver graft survival indicates that complement plays a role in hepatic xenograft rejection. Prolonged exposition to a hostile environment resulted in mild to extensive monocyte infiltration, whereas heart grafts demonstrated a more prominent granulocyte infiltration. In both grafts, more IgG than IgM deposition was noted.

Although CsA alone had no effect on hamster heart graft survival and CVF only prolonged it for a few days, their combination has been proven successful [14,26]. Van de Bogaerde et al. showed that about 20% of the heart grafts survived more than 100 days [13]. Scheringa et al. claimed an even higher portion [14]. Following the inability to ascribe a primary role to T-cells in concordant rejection, it is remarkably that rejection in CVF-treated recipients includes mechanisms that are sensitive to CsA. Nevertheless, this combination failed to prolong survival in our heart and liver transplant models. Histologically, liver grafts showed extensive monocyte infiltration, vessel and parenchymal damage, and extravasation of erythrocytes, indicating a

combined antibody-mediated and cellular rejection. Mild to massive IgM depositions, related to survival times, were found on hepatocytes and Kupffer-cells together with infiltrating macrophages. As complement levels were non-detectable, these results strongly suggest an ADCC.

Surprisingly, it was noted that long-term survival of hamster hearts, and possibly livers, was not achieved in the current model. CH50- and AH50-assays indicated that depletion was complete. Immunohistochemistry confirmed the absence of terminal complement components in these heart and liver graft recipients. A possible explanation is that the immune response capacity of the BN rat recipient is stronger than the rat strains, like Lewis and PVG, used by others [14,26]. Therefore, the partial inhibition of CsA of the antibody response and, consequently, ADCC, as suggested by Scheringa et al. [14], might not be that effective in our model. Hence, in a second set of experiments, we doubled the CsA-dosage. This combination therapy resulted in long-term survival of 50% of the heart grafts. However, after liver grafting, we were unable to keep the animals alive. This extremely high CsA-dosage resulted in enormous weight loss in all recipients. In combination with the surgical trauma related to liver transplantation, recipients died after a few days following serious emaciation.

In summary, it has not been previously reported that in concordant liver graft rejection, as in concordant heart graft rejection, complement plays an important role. In our model, the relatively high CsA-dosage combined with CVF was unable to prolong survival, compared to CVF alone. Histology indicated that ADCC plays a dominant role in rejection. Doubling the CsA-dosage resulted in 50% long-term heart graft survival, but toxic side effects leading to serious emaciation and death prevented liver recipient survival.

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Part B

Physiology in xenotransplantation

CHAPTER Seven

The fourth barrier

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Summary

At the entrance of a new era, clinical xenotransplantation is a valued and auspicious option in tackling the problem of donor shortage. Because of ethical and anatomical issues, 'farming' animals are considered the most favourable species for organ donation, but transplantation of their organs leads to a complex process of rejection. Mechanistically, three immunological barriers are distinguished: hyperacute rejection, delayed xenograft rejection and a subsequent cellular rejection. This review shall focus on problems regarding the fourth barrier, i.e. physiology in optional clinical settings and their corresponding animal models. Additionally, a fifth, microbiological, barrier is also being recognised.

Besides anatomical differences and posture, biochemical differences may have a severe impact on recipient survival. Disparity in blood components and electrolyte and other biochemical concentrations is easily detected between the species considered for xenotransplantation. Enzymes and hormones have complex routes of action, activation and inhibition and their molecular differences can impede function. Though infusion or medicine may correct certain imbalances in electrolytes and proteins, problems concerning complex interactions might be difficult to retrieve and solve. Experimentally, transplantation of discordant xenografts shows promising results, but the first physiological problems have already been detected. So, based upon the few experimental data available and the comparison of veterinary physiology, differences are expected between the organs grafted, regarding the possible occurrence of physiological problems. Moreover, precaution must be taken to extrapolate long-term survival, because of species specificity.

Introduction

As the outcome of transplantation is steadily improving itself, the indication for organ transplantation expands. This has already led to increasing numbers of patients awaiting transplantation. Yet, the rise in numbers of organs available for transplantation has reached its limits. Numerous initiatives, including presumed consent, are not sufficient to solve the expanding need for organs. To conquer this problem, the use of animal-derived organs, xenotransplantation, seems to be the most promising way to supplement or replace human allografts in the future.

Evolution led to a great variety of species that is still affected by hereditary and environmental influences. Because of this species variety, most phylogenetic characteristics, like genetic make-up, anatomy and physiology, form likely barriers in transplantation. Linnaeus, Lamarque and Darwin ordered the information about animals

and their relationship to man. Based on this classification, the phylogenetic distance between donor and host in xenotransplantation seems to be inversely related to the transplant survival time [1]. Transplantation between two species of one zoological family, for example between primate and man, usually results in acute cellular rejection, whereas between two families within an order, a mixed humoral and accelerated cellular rejection will follow. When the barrier of zoological orders is crossed, hyperacute rejection is seen, which is dominated by antibody-mediated mechanisms. Within this general pattern, however, lie many inconsistencies [2].

Primates and monkeys were first used as donors in clinical transplantation across a species barrier [3,4]. Whereas this model showed promising results, it will never offer the solution to donor shortage for a number of reasons. Most monkeys will not reach the size of an adult human; breeding larger animals will take decades [4]. More importantly, monkey organs carry a great risk of transferring specific pathogens to human [5] and primate reproduction for transplantation is considered unethical. The use of more distantly related 'farming' animals, like pig, sheep or cow, is less hampered by these problems.

To study xenotransplantation in an easier and faster way, small animal models have been developed mainly with the rat as organ recipient. The immunology of the rat has been subject of research for many years and has been characterised in detail, which indicated that processes of allograft rejection are similar in rat and human. Organ donors, like mouse, hamster or guinea pig, correspond to primates, monkeys and pigs, respectively for the human situation. In this way, clinical hypotheses can be tested promptly in relevant animal models.

Transplantation of organs, derived from distantly related, 'discordant', species, will lead to hyperacute rejection (HAR) resulting in total destruction of the graft in minutes to hours. Rapid progress in overcoming this first barrier by genetic engineering of the donor suggests that hyperacute rejection will not be a major issue [6,7]. Still, a second barrier, the "delayed xenograft rejection" (DXR) and a subsequent third, involving cellular rejection, will follow and have to be conquered. Besides the above-mentioned immunological barriers, a fourth obstacle of a different dimension can be defined. Evolution theory and veterinary physiology predicts that species specific physiology could be a major barrier. Additionally, a fifth barrier, implying the possibility of retroviral infection after grafting, might pose another threat to clinical xenotransplantation [8].

In the present review, I shall mainly focus on transplantation from 'farming' animals to man and the corresponding small animal models.

Xenograft immunology

As stated before, xenotransplantation is severely hampered by several rejection processes and the occurrence of these processes are related to the zoological relationship of the species used. In discordant grafting, it is known that, once hyperacute rejection is overcome, mixed cellular and humoral rejection will follow [9]. With increasing phylogenetic distance, it appears that the onset of rejection not only shortens, but that the different underlying processes, also become more complex. As the pig is the most likely donor in clinical xenotransplantation, three immunological barriers, namely HAR, DXR or "acute vascular rejection", and cellular rejection, will ensue.

Hyperacute rejection – the first barrier

During evolutionary development, antibodies, including preformed natural antibodies (PNAb), have evolved as one of the mechanisms of first-line host defence. PNAb exist in all vertebrates and are thought to be transferred from mother to child via placenta, colostrum or milk [10]. Other theories include their appearance as a result of a response to bacterial or cell fragment determinants [11,12]. The xenoreactive part of PNAb is the primary trigger in discordant xenograft rejection, most of them being IgM. The more distantly related the species, the higher the titres of the xenoreactive natural antibodies [13].

A major physiological difference already exists within the first barrier: i.e. complement disparity. The major effector in hyperacute rejection, complement is activated by either deposited antibodies or by itself. This activation is not controlled by regulators, like decay accelerating factor and membrane cofactor protein, because of species specificity. Incorporating recipient-specific complement regulators by genetic engineering or complement depletion results in prolonged survival [14,15]. Yet, several other processes remain active. (For review see [16,17].)

Delayed xenograft survival/Acute vascular rejection – the second barrier

Mastering HAR still results in rejection of grafts after several days [18]. Complement regulators or depleting agents do not obstruct processes, like adhesion molecule expression, interleukin production and antibody binding. Interactions of the host immune system with molecules produced by these processes are also species specific [19-21]. Infiltration of monocytes, macrophages and NK-cells, probably initiated by bound antibodies and expression of specific endothelial cell epitopes, coincides with the perceived delayed rejection. The damage, like in HAR, is most probably caused by molecular incompatibilities. Disparity between NK-cell inhibiting responses as well as the disparity of the (monocyte synthesised) complement components and its regulators could well be responsible for the subsequent impairment of the organ [22-24].

Whereas high dosage immunosuppressives alone lead to minimal prolongation of survival, the combination with incorporation of host regulators could be an improvement. However, regarding the current status of genetic engineering, complex and manifold genetic inclusions of different molecules may not be the solution to overcome the complex process of delayed xenograft rejection. (For review see [25,26].)

Cellular rejection – the third barrier

Until now, nothing is known about this third barrier in *in vivo* discordant xenograft rejection. Historically, the cellular response was thought to be not that important because of the disability of T-cell receptors to recognise non-self MHC and the disparity of molecular interactions involved. Diminished frequencies of responder cells as well as reduced cytokine interactions indicated this third barrier would not be a major issue [27-29].

However, more recent studies demonstrate that several T-cell interactions are intact in the human anti-pig response [30,31]. Moreover, Murray et al. reported that the human anti-pig cellular response is mediated both by direct and indirect pathways of recognition [32].

How the cellular rejection arises *in vivo* is still unknown. However, it appears that the responses seen *in vitro* require immunosuppressive regimens at least as potent as those used in allografting [33].

Anatomy

Mammalian species differ in anatomy as a consequence of evolution. As by definition species barriers are crossed in xenotransplantation, existing anatomical differences, like organ size and position of veins, arteries and other conducting vessels, might impede successful transplantation. Breeding can change animal and, consequently, organ size, but their structure and function remain unaltered [34]. The pig has a remarkably similar anatomy and shares a variety of characteristics with man including size, haemodynamics and coronary artery distribution [34]. In small animal surgery, size breeding has not been done and as rat recipients weigh between 200 and 300 grams at the time of transplantation, adult hamsters and very young guinea pigs are required when graft size is important. Nevertheless, the differences in organ size sometimes reach 30%. Experimentally, heart graft size is less important for these are always placed heterotopically in the abdomen.

The posture of donor species might influence the future function of transplants when man is the recipient. Humans have an upright position, which is shared by few other species, like primates and kangaroos. Posture, erect or horizontal, influences blood circulation and function of organs. For example, horizontal bearing animals have

enhanced upper lung function, whereas humans have a more abdominal ventilation. In an upright position, blood pressure differences are large between and even within organs. After xenogeneic lung transplantation, ventilation could be hampered or, moreover, the recipient could possibly die due to oedema.

Comparative physiology [35-38]

If one day xenografted organs will be accepted immunologically, nothing is known about the compatibility of the physiology of the organs and their host. Communication pathways as they exist in all mammals are controlled on several levels by physical and (bio-) chemical processes. These processes include pressures, gradients and binding properties, but also inhibitors, activators, receptors and their underlying (feedback) mechanisms. Most basic metabolic characteristics are similar in different mammalian species, however, it is the organisation of communication and its controllability that will determine the final outcome of xenotransplantation.

Hence, apart from endless *in vitro* testing to exclude species specificity for the molecule of interest, comparison of physiological parameters is the only sensible thing to do. Comparative physiology can indicate possible differences that might interfere with survival of the transplanted organ or even the host.

Between man and closely related species, like primate and ape, the known physiological parameters, i.e. biochemical concentrations, are rather alike, but as soon as the zoological barrier is crossed, major differences can be found. No long-term survival of discordant xenogeneic transplants has ever been reported most probably due to immunological interference, although physiological incompatibilities can not be ruled out.

The rat transplantation models with hamster or guinea pig as organ donor are frequently used to study the first three barriers. However, these models might not be relevant to study the fourth barrier. The physiological differences between human and primates are presumably model dependent and can, therefore, not be studied in the rodent xenotransplantation. Nevertheless, survival times of specific transplantation models and the comparison of physiological characteristics of both species could give information about the lacked ability to reach prolonged survival or 'vain' physiological parameters. Long-term hamster organ survival has proven possible with relatively mild immunosuppressive protocols [39,40]. Therefore, it is highly unlikely that if differences in physiology exist, they are relatively unimportant for the final survival of the recipient. Discordant grafting, so far, is rather unsuccessful. Indeed physiology could play an important role and could contribute to the complexity of xenotransplantation.

Haematology

The function of organs depends primarily on the blood circulation. Total protein contents and the size and amount of the blood components determine the viscosity of the blood. The viscosity of human blood is lower than that of pigs, but higher than that of other farming animals, like cow, sheep and horse. In clinical transplantation, improved haemoperfusion can be expected when the pig is used as a donor. However, hematocrit comprises 30% of the blood volume in pigs and 40% in man. Moreover, human erythrocytes are certainly the largest of all mammals of interest for clinical xenotransplantation (see Table 1). Increased size of red blood cells could lead to mechanical interference in the microcirculation and, therefore, cause damage to the transplant as well as the blood components.

Blood groups of most domestic animals seem to resemble more the Rhesus, than the ABO system in humans and primates [41]. In allografting, ABO matching is standard procedure, because of the induction of severe rejection by ABO-antibodies. However, it has been reported that human isohaemagglutinins seem to have no influence in the rejection of pig organs [42].

Blood pressure maintains the flow of nutrients and oxygen to organs and tissues. Decreased pressure leads to hypoxia and loss of conscience; hypertension would finally result in organ damage. When comparing rodent parameters, major differences can be seen. Guinea pig blood pressure is much lower than in rat, mouse and hamster. After xenotransplantation, guinea pig organs will sustain persisting elevated blood pressure

Table 1. *Blood parameters of man, animals considered for clinical xenotransplantation and rodents most commonly used in xenotransplantation* [35-38,62].

Species	Viscosity (10^{-3}Pa s)	Total protein (g/100ml)	RBC (10^6/µl)	WBC (10^3/µl)	RBC diameter (µm)	SBP/DBP (mmHg)
Man	4.7	7.3	5.0	7.0	7.2	120/80
Pig	5.9	7.3	6.5	12.0	6.1	130/90
Sheep	4.3	8.0	10.0	8.0	5.1	135/90
Cow	4.6	7.5	6.0	8.0	5.7	145/90
Rat	-	6.3	7.3	9.8	6.2	120/60
Mouse	-	6.4	8.6	9.2	5.7	110/70
Hamster	-	6.0	7.2	6.3	5.7	150/100
Guinea pig	-	5.4	5.4	9.9	7.0	85/56

RBC: red blood cell, WBC: white blood cell, SBP: systolic blood pressure, DBP: diastolic blood pressure.

leading to oedema and possibly bleeding. No differences exist regarding blood pressure of animals considered for clinical xenotransplantation and man. Nevertheless, as mentioned before, blood pressure differences in man are much higher than in pig because of its vertical posture. It remains to be seen whether xenografted organs can adapt to such a situation.

Blood parameters of man, animals considered for clinical xenotransplantation and rodents most commonly used in xenotransplantation are listed in Table 1.

Biochemistry

Enormous amounts of biochemical elements are produced in order to maintain homeostasis, species evolution and survival. Most constituents are essential for life; some are less elementary.

Electrolytes

Minerals perform several tasks, which comprise maintenance of cellular membrane electrical activity, cellular processes, including muscle contractions and enzymatic reactions, and skeletal construction. They are also important for osmolarity, acidity and volume of body compartments. The kidney regulates the serum ions by glomerular filtration, tubular resorption and tubular secretion. Dietary habits of a particular species ascertain to a large extend these processes. Clinical xenotransplantation of chimpanzee kidneys led to survival times up to 9 months, however, marked diuresis was often seen [43,44]. The urinary loss of sodium and, particularly, potassium often appeared to be large in patients receiving xenografts [45]. As chimpanzees take diets high in potassium, this might explain the function of these kidneys [44]. Electrolyte concentration differences between man and other possible organ donors are rather large (see Figure 1). Between man and pig, the most obvious organ donor, only sodium and chloride levels seem to be identical. Especially calcium and phosphorus are notably lower in man than in pig. Upon kidney transplantation, higher concentrations of both ions would be established. If the solubility value of calciumphosphate is crossed, a situation that could easily occur, salt will deposit in bones but also in other tissues.

The main osmotic intracellular activity is regulated by potassium. Changes in extracellular potassium levels can be extremely dangerous. Elevated or decreased potassium levels lead to reduced muscle strength and possibly death. In rats, the mean potassium level is 5.9 mEq/l; in guinea pigs and hamsters, potassium levels are 7.4 and 4.1 mEq/l, respectively. Yet, long-term survival of hamster kidneys transplanted in rats, although rather difficult to achieve, is possible [46]. This indicates that, if lower serum potassium levels are established in this model, some degree of adaptation takes place, which might also occur for other electrolytes. Upon clinical xenotransplantation, this might prove beneficial.

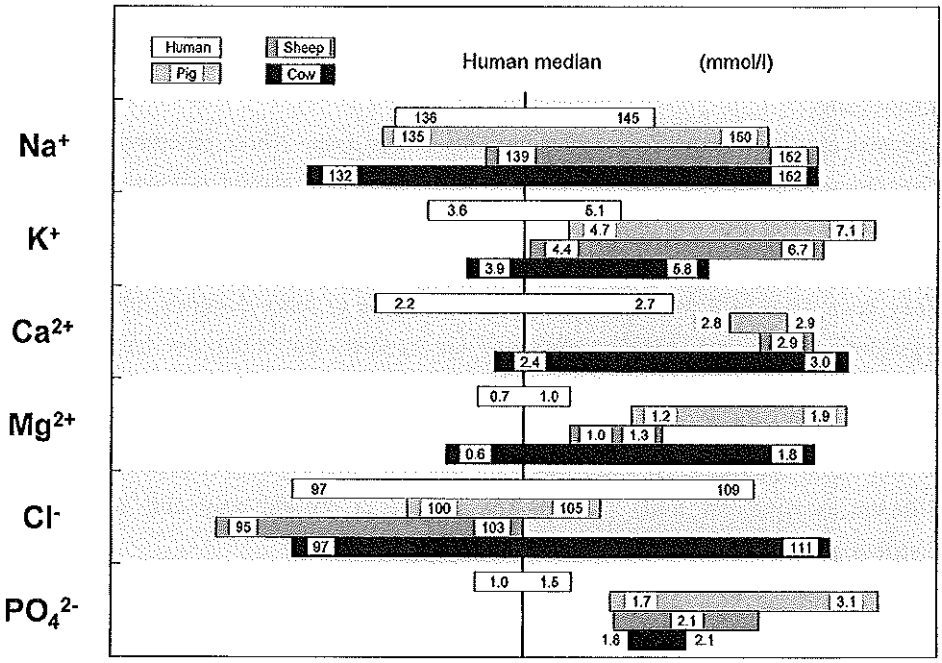

Figure 1. *Extracellular electrolyte concentrations of man and animals considered for clinical xenotransplantation* [37].

Hormones and enzymes

Hormones regulate metabolism, growth and maturation of the organism, organ function necessary for reproduction, and 'internal environment', like blood circulation, acidity and water and electrolyte housekeeping. The range of hormones is similar in all vertebrates, with some species possessing specific molecules. As stated earlier, hormones will only function normally, if the physiological process is regulated by factors, like hormone releasing factor and hormone inhibitors and their releasing factors. Growth hormone, which differs between man and pig by 19% and between man and whale by 34%, indicates that hormones can be rather species specific regarding their molecular structure [47]. Incorporation of human growth hormone genes in mice leads to the development of giant mice [48]. In pig, it seems that livers metabolise human growth hormone in a similar way as the porcine variant [49].

Patients suffering from diabetes mellitus type I require chronically administered insulin to regulate their glucose levels. Nowadays, recombinant human insulin is used, but porcine insulin was also able to regulate these levels. Yet, pig erythropoietin failed to be effective upon pig to monkey kidney transplantation and led to anaemia, which was prevented by treatment with human recombinant erythropoietin [50]. It remains,

therefore, questionable whether monitoring hormone levels is of any value. Serum levels will not reveal receptor-linked incompatibility and, consequently, hormonal functionality.

Enzymes catalyse various processes within the cell or tissue. These processes may even experience more species specificity than hormones. One should not only take into account the 2500 different kind of enzymes produced by the liver, but also their intrinsic variance. It is postulated that enzymes already existed in the first cellular organisms. They have developed from purely digestive elements to more complex, physiological regulators. Even tissue-specific variants of one enzyme exist within one individual, for example lactate dehydrogenase and alkaline phosphatase. During ontogeny, this specificity can even change. Certain foetal tissue (iso-)enzymes barely function in adults of the same species [51]. Enzymes, like catalase and carboxylesterases, appear in different isotypes. In some species, one isotype is present only and activity and distribution varies markedly between species [52].

After xenogeneic liver transplantation, enzymatic blood constituents, like complement components and coagulation factors change rapidly to donor-type proteins [4,53,54]. At the moment of transplantation, these constituents are of recipient origin, which could prove an obstacle in xenotransplantation. Jurd et al. reported activation of haemostasis *in vitro* in the pig to human xenotransplantation model [55]. However, pig liver perfusion with human blood to overcome hepatic coma was not hampered by haemostatis with only local anticoagulation [56].

Furthermore, liver synthesised choline esterase partially regulates the activity of the neurotransmitter acetylcholine. It mediates various processes, like elevated secretion by glands of the digestive system and vasodilatation. In rats, median choline esterase level is 98 U/l. Median hamster choline esterase levels are about 3 times higher, 309 U/l, whereas choline esterase levels in guinea pigs are 10 times higher, 1039 U/l. Indeed, after guinea pig liver transplantation, choline esterase levels showed a 5- to 7-fold increase when compared to the rat recipient value [57]. The long term effect of chronically reduced acetylcholine neurotransmission is unknown.

Other biochemical constituents

Quantitatively, the most important serum-protein is albumin. Albumin maintains the colloid-oncotic pressure of the serum and represents the main carrier molecule of hormones and other biochemical constituents, like anions, fatty acids, metabolites and drugs. Albumin levels differ significantly between man and pig and even between man and primate. In 1992, a 35-year old male with hepatitis underwent orthotopic liver transplantation with a baboon as donor [4]. For the first two postoperative weeks, human albumin was supplemented. Thereafter, the serum protein pattern of the recipient rapidly approached that of the baboon. The low serum albumin produced by the baboon

xenograft was well tolerated [4]. Rat albumin ranges from 3.8 to 4.8 g/100ml, whereas guinea pig albumin concentrations vary from 4.6 to 6.2 g/100ml. Hamster albumin values range from 2.6 to 4.1 g/100ml. The effect of extreme differences in albumin concentration is unknown, but it could influence blood osmolarity leading to oedema or dehydration after xenotransplantation. None of these phenomena, however, have been reported in the hamster to rat liver transplantation model. In addition, human individuals exist, which suffer from analbuminemia (idiopathic hypoalbuminemia) [58]. This congenital anomaly is characterised by very low levels of this protein. Except for moderate oedema in only a third of the reported cases, serious consequences of the lack of albumin are absent [58]. Upon clinical liver xenografting, a weaning period might be preferred with controlled infusions of human albumin instead of an abrupt change to donor levels.

Serum cholesterol, synthesised in the liver, is an important precursor for steroid hormones, which are essential for homeostasis, normal reproduction and response to stressful situations. In pig, cholesterol is extremely low, compared to the human level (45 mg/100ml vs. 200 mg/100ml). This indicates that grafting of pig organs, other than liver, might eventually lead to accelerated formation of arteriosclerotic lesions. Levels of cholesterol vary between 28 and 76 mg/100ml in rats. Mean hamster cholesterol concentration is about 130 mg/100ml. After hamster liver grafting, circulatory problems could rise in time. Effects, if any, will only be detected after a very long-term follow-up.

Glucose, regulated by consumption and formation, is the central energy carrier for various biochemical processes. Blood glucose levels in man (80 to 100 mg/100ml) are different from sheep and cow (35 to 60 mg/100ml) but similar to pig. High blood sugar levels could be disastrous to sheep or cow kidney transplants, like in human diabetes mellitus.

Clinical baboon liver transplantation resulted in a patient survival of 26 and 70 days [4,59]. Patient death followed biliary stasis with consequent diffuse damage of intrahepatic ducts [4]. The biliary damage was already diagnosed 12 days after grafting with no signs of rejection [60]. Although technical, immunological or even chemical injury has been suggested as a possible cause [4], physiology might also be involved.

Conclusion

Xenotransplantation could be the possible solution to donor shortage. Immunological, physiological and infectious barriers impede a fast incorporation of this technique in clinical practice. As porcine hearts already survive more than 3 months in monkeys, hyperacute rejection will not be a major barrier anymore [7]. Nevertheless, high dosages of immunosuppression that can not be tolerated in the clinical situation so far are needed

to keep organs functional. Other barriers, like delayed xenograft rejection and possibly cellular rejection, are still rather unenlightened areas.

Rodent models offer the opportunity to fully investigate rejection patterns. In certain hamster to rat transplantation models, long-term survival is relatively easy to establish [40,61]. Thus, the physiological differences indicated are of minor importance for maintaining life. It can, however, not be ruled out that this is model specific. Moreover, 'long-term' is mostly referred to as more than 100 days survival, which could be 'short' from a physiological perspective. Discordant guinea pig to rat transplantation is less successful. In this model, all solid organ graft survival times, published so far, do not exceed 6 days [15,57]. Liver recipients, in contrast to heart graft recipients, die without signs of rejection, indicating a physiological incompatibility [57]. Exposition of the grafted organs to continuous elevated blood pressure and decreased potassium levels could well lead to organ damage and, therefore, reduced survival.

Taking notice of the different levels of possible molecular and physiological incompatibilities, variable outcomes for distinct organ transplants might be expected. A heart depends on a rather small number of substances to continue its function. Electrolyte disturbances will have a major impact, whereas the influence of other biochemical elements will be rather small. This is in contrast to the liver, which is the most important metabolic organ of the body and produces numerous substances. Upon transplantation, it is likely that one or more of these substances are species specific in such a way that their metabolism is heavily diminished or blocked. Depending on the substance(s), a severe impairment of life or even incompatibility could be the result. The kidney, as a clearing organ, but also as a small metabolic organ, holds a position somewhere between the heart and liver concerning the possible occurrence of physiological problems after transplantation. Supplementing life-essential hormones or proteins might prove a solution in kidney grafting in contrast to liver grafting.

Experimental xenotransplantation of pig organs to monkeys may show promising results, however, precaution must be taken to extrapolate 'success' even if long-term survival is possible. It should be emphasised that although physiology of the monkey may be similar to human, it is definitely not the same.

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Conclusions

Summary and general discussion
Samenvatting en algemene discussie

Summary and general discussion

Up to now, transplantation is the only therapy for patients with endstage liver disease. In the 70's, patient survival increased due to the introduction of cyclosporine A (CsA). Since then, many pharmaceutical companies have entered the transplantation market and several new target-specific immunosuppressive drugs have been introduced. Together with new insights in individual transplant rejection, future outcome of clinical grafting will definitely improve further. This will also broaden the indication for transplantation. As the increase in supply of donor organs stagnates, waiting lists are growing. Consequently, patients die awaiting transplantation. Initiatives to overcome donor shortage, like the introduction of presumed consent, will not solve this problem. One possibility that offers an almost infinite supply of donor organs is xenotransplantation, transplantation of organs or tissues across a species barrier. The pig is considered the most likely species for transplantation purposes, because it procreates rapidly and its use is less hampered by ethical issues than the use of primates. However, xenografting of pig organs results in hyperacute rejection (HAR) caused by complement activation and deposition of preformed xenoreactive natural antibodies. To study this immunological process, the guinea pig (GP) to rat heart transplantation model is often used.

In clinical allografting, livers transplanted across an ABO mismatch or T-cell positive warm crossmatch are neither hyperacutely rejected nor demonstrated histological abnormalities. To investigate a potential immuno-privileged position of the liver in xenotransplantation, the experiments using a discordant rodent model described in this thesis were initiated.

Mastering xenogeneic orthotopic liver transplantation

The technique of orthotopic liver transplantation (OLT) had to be mastered to study the susceptibility to antibody-mediated rejection of liver xenografts. In the **chapter** dealing with **Technical aspects of experimental orthotopic liver transplantation in the rat,** we describe this elaborate technique in full detail. The cuff-technique was adopted, whereas rearterialistation of the hepatic graft was not applied. This technique resulted in indefinite survival in iso- and allografts. It must be stated that OLT requires extensive microsurgical experience and patience.

In **chapter 1,** OLT was performed to study the applicability of this technique in xenografting. The feasibility of OLT in the GP to rat model is largely unknown. Hence, liver transplantation was carried out in the hamster to rat model, a combination extensively studied in xenogeneic heart transplantation. Early death of the liver

recipients was encountered when immunosuppression included irradiation of the recipient. FK506-treatment resulted in prolonged survival. We concluded that the combination of surgical trauma related to the transplantation procedure and the effect of irradiation had a larger impact in liver xenografting than in heart grafting. Nevertheless, it was realised to reproduce prolonged liver graft survival as was found by others. Thus, in the hamster to rat model, liver transplantation is a difficult but adequate model to study xenotransplantation.

Discordant xenografting

After it was demonstrated that xenogeneic liver transplantation was technically feasible, discordant GP to rat grafting was performed. The relative protection of the liver for HAR was investigated as described in **chapter 2**. To our surprise, all GP liver recipients died with functioning grafts, even though 2 animals survived for 99 and 147 hours after complement depletion with cobra venom factor (CVF). No (immuno-) histological evidence was found for rejection. We concluded that discordant liver graft recipients died because of non-immunological problems. Although not demonstrated in our study, other studies suggest that reperfusion and ischaemia lead to the release of vasoactive substances that causes irreversible shock. On the other hand, CVF-toxicity might play a role.

Discordant grafting in an intrinsic complement deficient rat would elucidate the potential hepatotoxic role of CVF in the rat recipient. In **chapter 3** therefore, the PVG/c^- rat was analysed for its status of complement deficiency and its suitability for liver transplantation. By performing liver transplantation between PVG/c^- rat (C6 deficient and partial C2 deficient) and its "syngeneic" 'complement-sufficient' PVG/c^+ counterpart, it was concluded that C6 deficiency is caused by anti-C6 antibodies. We deduced that in the PVG/c^- rat complement activation can still occur after xenotransplantation but that no terminal complement complex can be formed. Therefore, the PVG/c^- rat seemed suitable for discordant transplantation.

In **chapter 4**, the PVG/c^- rat and the partial auxiliary liver transplantation technique were used to study CVF-toxicity and primary non-function of the GP liver. In both studies early recipient death was encountered that led to the conclusion that neither CVF-toxicity nor primary non-function was responsible for early failure. Also, the PVG/c^- rat was considered unable to withstand discordant grafting in combination with the surgical trauma related to liver transplantation. The results described in **chapter 2** and **chapter 4** suggest that the GP liver is sensitive to handling and ischaemia/reperfusion injury. Transplantation and perfusion experiments carried out in the GP confirmed that GP liver grafts are very sensitive to injury mediated by

ischaemia/reperfusion and surgical trauma. This is probably because of the release of vaso- and immunoactive substances.

Susceptibility to antibody-mediated rejection

As the reduced susceptibility to antibody-mediated rejection of the liver could not be investigated in the discordant GP to rat model, discordancy was mimicked in a sensitised hamster to rat model. By using different immunisation protocols, predominance of either IgG or IgM antidonor antibodies was created. Transplantation of hamster heart or liver in both sensitisation models revealed a difference in rejection pattern between both organs. As described in **chapter 5**, a predominance of IgG over IgM was found regarding the HAR of liver grafts. This difference in rejection was not found for heart grafts.

Besides HAR, the liver might also be less susceptible to antibody dependent cell-mediated cytotoxicity (ADCC). ADCC may arise after complement depletion and inhibition of T-cells. In **chapter 6**, it is demonstrated that hamster heart and liver grafts are rejected by ADCC. Long-term heart survival was achieved by an extreme immunosuppressive regimen. As all liver graft recipients suffered from early death, no difference could be found between heart and liver grafts regarding the possible ADCC rejection.

Comparative physiology in xenotransplantation

The evolution theory and veterinary physiology predict that, besides HAR, delayed xenograft rejection and T-cell mediated rejection, species-specific physiology could be an important fourth barrier. In **chapter 7** denoted as **The fourth barrier**, a review is presented about physiological differences between species considered for clinical xenotransplantation and man and between species in the relevant rodent models. Differences in physiology in the relevant pig to cynomolgus monkey model have already been detected. It is the opinion of the authors that xenografting of the liver, the most important metabolic organ, incorporates a high risk of physiological impairment for the recipient/patient.

Concluding remarks

In the present thesis, research was focussed on the potentially reduced susceptibility to antibody-mediated rejection of liver grafts in xenotransplantation and the physiological compatibility of hepatic xenografts.

After establishing the xenogeneic OLT technique, the reduced susceptibility of liver xenografts to antibody-mediated rejection was studied in the GP to rat model. To our dismay, liver recipients died with functioning, non-rejected grafts. No classical signs of HAR were found in the GP liver transplants. Liver grafting in the intrinsic complement deficient PVG/c^{-} rat and application of the partial auxiliary liver transplantation technique revealed that neither CVF-toxicity nor primary non-function of the GP liver were responsible for the early deaths encountered. Both syngeneic GP liver transplantation and GP isolated liver perfusion resulted also in early death of the recipients. Therefore, it is concluded that GP liver is unsuitable for transplantation because of its sensitivity to surgical trauma and ischaemia/reperfusion injury.

In the GP to rat liver transplantation model, removal of the released vasoactive and immunoactive substances may be less rapidly because of the impaired liver function. However, in the heart transplantation model in the same species combination, where a normal hepatic function is present, reperfusion of the cardiac transplant still leads to early recipient death in up to 40% of the cases. Thus, it is hypothesised that not only liver function as such is an important factor, but also the amount of vasoactive and immunoactive substances released by the transplanted GP organ. So, GP organs in general are sensitive to surgical trauma and ischaemia/reperfusion injury induced in transplantation.

Because it was impossible to investigate the assumed immuno-privileged position of the GP liver, the study of this phenomenon was carried out in the "pseudo-discordant" hamster to sensitised rat model. We demonstrated that HAR in hepatic grafting was mediated by IgG but not by IgM. This predominance did not exist for heart grafts. In normal concordant hamster heart and liver transplantation, survival times of liver grafts are significantly longer than those of heart grafts. The elicited antibody response is initially of the IgM-type after which it switches to an IgG-type. No difference in immunogenicity exists between heart and liver grafts. In the GP to rat model, preformed natural antibodies are mainly IgM. In this model, heart grafts are hyperacutely rejected, whereas liver recipients die with non-rejected grafts. IgM depositions were marginal or not present. Therefore, it is concluded that in concordant as well as discordant xenotransplantation the liver graft holds an immuno-privileged position compared to heart grafts. The liver, but not the heart, is less susceptible to IgM-mediated rejection. In humans, the preformed antibodies are also mainly IgM-type. The absence of (hyper-)acute rejection after clinical use of pig liver in extracorporeal perfusion explains en further demonstrates the finding that the xenogeneic liver is less susceptible to IgM-mediated rejection.

No firm conclusions can be drawn regarding the reduced susceptibility of liver xenografts toward antibody-mediated cellular rejection. It seems, however, that the liver

is not immuno-privileged with regard to antibody-mediated cellular rejection as it rejects with the same pace as heart grafts.

Physiological compatibility of discordant GP livers in rat recipients could not be investigated due to the absence of long-term graft survival. However, comparing veterinary physiology revealed major differences in haematology and in electrolyte and enzymatic levels. From rodent physiology, it is deduced that GP to rat organ grafting will not result in long-term survival, due to haemodynamical and biochemical incompatibilities. Comparison of the better-documented pig and human physiology indicates large differences between both species. Organ transplantation of to human closely related species like baboon and chimpanzee already resulted in physiological alterations. These findings highly suggest that species-specific physiology is an important barrier in xenotransplantation.

It is unlikely that xenogeneic liver transplantation will become clinical practice without any additional genetic modification to overcome immunological barriers. Moreover, further attention should be focussed on physiological imbalances in liver function between species, especially between pig and man. Although the liver is immuno-privileged, it is our believe that xenogeneic liver transplantation will not become a life-supporting procedure in the near future, because of the specific limitations, mentioned above. It is, furthermore, conceivable that because of the progress in cloning techniques and artificial organ development, these applications will outdo xenotransplantation of the liver.

Samenvatting en algemene discussie

Tot op heden is transplantatie de enige therapie voor patiënten met terminaal leverfalen. Na de introductie van cyclosporine A (CsA) in de jaren 70 is de overleving van patiënten na transplantatie toegenomen. Sindsdien zijn er vele farmaceutische bedrijven op de transplantatiemarkt verschenen en zijn diverse nieuwe doel-specifieke immunosuppressiva geïntroduceerd. Mede vanwege de nieuwe inzichten op het gebied van individuele orgaan afstoting, zal de uitkomst van transplantatie in de toekomst zeker verbeteren. Dit zal eveneens leiden tot een verbreding van de indicatie voor transplantatie. Omdat de toename van het aanbod van orgaandonoren stagneert, worden de transplantatiewachtlijsten steeds langer. Als gevolg hiervan sterven patiënten, wachtend op een transplantatie. Initiatieven om het donortekort op te heffen, zoals het geen-bezwaar-systeem, lijken niet voldoende om dit probleem te boven te komen. Een alternatieve, bijna onuitputtelijke bron van donororganen, is xenotransplantatie, orgaan- en weefseltransplantatie over een speciesbarrière. Het varken wordt beschouwd als de meest voor de hand liggende species voor transplantatiedoeleinden. Dit vanwege zijn snelle voortplanting en omdat het gebruik van varkens minder ethische problemen met zich mee brengt dan het gebruik van primaten. Xenotransplantatie van varkensorganen resulteert echter in hyperacute afstoting (HAR) wat wordt veroorzaakt door binding van voorgevormde antilichamen en complementactivering. Voor het bestuderen van dit immunologische proces wordt vaak het cavia naar rat model gebruikt.
In klinische allotransplantatie is gevonden dat levers noch hyperacuut afstoten, noch histologische afwijkingen vertonen wanneer ze getransplanteerd worden over een ABO-barrière of met T-cel positieve warme crossmatch. Om een potentieel immunologisch bevoorrechte positie van de lever in xenotransplantatie te bestuderen, werden experimenten geïnitieerd, gebruik makend van een discordant knaagdier model beschreven in deze thesis.

Het bekwamen in xenogene orthotope lever transplantatie

Om de verminderde gevoeligheid van xenogene levertransplantaten te kunnen bestuderen, was het noodzakelijk zich de techniek van orthotope levertransplantatie (OLT) aan te leren. In het **hoofdstuk** met als titel **Technical aspects of experimental orthotopic liver transplantation in the rat** wordt deze techniek uitvoerig beschreven. De cuff-techniek werd overgenomen, terwijl het herstel van de arteriële bloedvoorziening van de lever achterwege werd gelaten. Deze techniek resulteerde in permanente overleving van iso- en allotransplantaten. Het moet worden vermeld dat OLT een uitgebreide microchirurgische ervaring en geduld vereist.

In **hoofdstuk 1** werd OLT verricht om de toepasbaarheid van deze techniek te bestuderen bij xenotransplantatie. De haalbaarheid van OLT in het cavia naar rat model is echter grotendeels onbekend. Daarom werd levertransplantatie uitgevoerd in het hamster naar rat model, een intensief bestudeerde combinatie in xenogene harttransplantatie. Geconstateerd werd dat leverrecipiënten vroeg overleden, wanneer bestraling onderdeel uitmaakte van de immunosuppressie. FK506-behandeling resulteerde in verlengde overleving. We kwamen tot de conclusie dat de combinatie van chirurgisch trauma, gerelateerd aan de transplantatieprocedure, en het effect van bestraling een grotere impact hadden op xenogene levertransplantatie dan op harttransplantatie. Desondanks werd verlengde overleving van de lever gerealiseerd, zoals ook door anderen gevonden was. Hamster naar rat levertransplantatie is daarom een moeilijk maar geschikt model om xenotransplantatie te onderzoeken.

Discordante xenotransplantatie

Nadat was aangetoond dat xenogene levertransplantatie technisch haalbaar was, werd discordante cavia naar rat transplantatie uitgevoerd. De relatieve bescherming van de lever tegen HAR werd onderzocht, zoals beschreven in **hoofdstuk 2**. Tot onze verbazing overleden alle leverrecipiënten met een functionerend transplantaat, ondanks dat 2 dieren 99 en 147 uur overleefden na complementdepletie met cobra gif factor (CVF). Er werd geen (immuun-)histologisch bewijs gevonden voor afstoting. Wij concludeerden dat discordante levertransplantaat ontvangers overleden als gevolg van niet-immunologische problemen. Alhoewel datgene niet kon worden aangetoond in ons experiment, suggereren andere studies dat reperfusie en ischemie leiden tot vrijmaking van vasoactieve stoffen, welke irreversibele shock veroorzaken. Aan de andere kant zou CVF-toxiciteit een rol kunnen spelen.

Discordante transplantatie in een intrinsiek complementdeficiënte rat zou de potentiële hepatotoxische rol van CVF in de ratontvanger kunnen verhelderen. In **hoofdstuk 3** werd daarom de PVG/c$^-$ rat geanalyseerd op zijn complementdeficiënte status en zijn geschiktheid voor levertransplantatie. Met behulp van levertransplantatie tussen de PVG/c$^-$ rat (C6 deficiënt en partiële C2 deficiëntie) en zijn "syngene" 'complementsufficiënte' PVG/c$^+$ tegenhanger, werd geconcludeerd dat C6-deficiëntie wordt veroorzaakt door anti-C6 antilichamen. Wij deduceerden dat in de PVG/c$^-$ rat nog steeds complementactivering kan optreden na xenotransplantatie, maar dat er geen terminaal complementcomplex gevormd kan worden. De PVG/c$^-$ rat leek daarom geschikt voor discordante transplantatie.

In **hoofdstuk 4** werden de PVG/c$^-$ rat en de partiële auxiliare levertransplantatie techniek gebruikt om CVF-toxiciteit en 'primary non-function' van de cavialever te

onderzoeken. In beide studies werd vroege ontvangerdood gevonden wat leidde tot de conclusie dat noch CVF-toxiciteit, noch 'primary non-function' verantwoordelijk was voor het vroege falen. Tevens bleek de PVG/c⁻ rat niet in staat weerstand te bieden aan discordante transplantatie in combinatie met het chirurgisch trauma gerelateerd aan levertransplantatie. De resultaten beschreven in **hoofdstuk 2** en **hoofdstuk 4** suggereerden dat de cavialever gevoelig is voor hantering en ischemie/reperfusie schade. Transplantatie en perfusie experimenten uitgevoerd in de cavia bevestigden dat cavialevers erg gevoelig zijn voor schade gemedieerd door ischemie/reperfusie en chirurgisch trauma. Dit wordt mogelijk veroorzaakt door het vrijkomen van vaso- en immunoactieve stoffen.

Gevoeligheid voor antilichaamgemedieerde afstoting

In het discordante cavia naar rat model kon de verminderde gevoeligheid van de lever voor antilichaamgemedieerde afstoting niet worden onderzocht. Discordantie werd daarom nagebootst in een gesensitiseerd hamster naar rat model. Predominantie van óf IgG óf IgM antidonor antilichamen werd gecreëerd, gebruik makend van verschillende immunisatie protocollen. Transplantatie van hamsterhart en -lever in beide sensitisatie modellen onthulde een verschil in afstotingspatroon tussen beide organen. Zoals beschreven in **hoofdstuk 5** werd er een predominantie gevonden van IgG ten opzichte van IgM wat betreft HAR van de lever. Dit verschil in rejectie werd niet gevonden bij harttransplantaten.

Naast HAR zou de lever ook minder gevoelig kunnen zijn voor antilichaamafhankelijke celgemedieerde cytotoxiciteit (ADCC). ADCC kan optreden na complementdepletie en inhibitie van T-cellen. In **hoofdstuk 6** is aangetoond dat hamsterhart en -lever transplantaten worden afgestoten door ADCC. Lange-termijn overleving van het hart werd bewerkstelligd door een extreem immunosuppressief regiem. Omdat alle leverrecipiënten vroeg overleden, kon er geen verschil worden aangetoond tussen het hart en de lever voor een mogelijke ADCC afstoting.

Vergelijkende fysiologie in xenotransplantatie

De evolutie theorie en veterinaire fysiologie voorspellen dat speciespecifieke fysiologie, naast HAR, vertraagd xenogene afstoting en T-cel gemedieerde afstoting, een belangrijke vierde barrière kan zijn. In **hoofdstuk 7** met als titel **The fourth barrier** wordt een overzicht gepresenteerd van de fysiologische verschillen tussen species die in aanmerking komen voor klinische xenotransplantatie en de mens en tussen de species in de relevante knaagdier modellen. Er zijn reeds fysiologische verschillen in het relevante

varken naar cynomolgus aap model gevonden. Het is de mening van de auteurs dat xenotransplantatie van de lever, metabool het meest complexe orgaan, een groot risico van fysiologische verslechtering van de ontvanger/patiënt met zich meebrengt.

Slotoverwegingen

In deze thesis was het onderzoek gericht op de mogelijk verminderde gevoeligheid van de lever voor antilichaamgemedieerde afstoting in xenotransplantatie en de fysiologische compatibiliteit van xenogeen getransplanteerde levers. Na de ontwikkeling van de xenogene OLT techniek werd de verminderde gevoeligheid van xenogene levertransplantaten voor antilichaamgemedieerde afstoting onderzocht in het cavia naar rat model. Tot onze schrik overleden de leverrecipiënten met functionerende, niet afstotende transplantaten. Er werden geen klassieke tekenen van HAR gevonden in de getransplanteerde cavialevers. Levertransplantatie in de intrinsiek complementdeficiënte PVG/c$^-$ rat en de toepassing van de partiële auxiliary levertransplantatie techniek wees uit dat noch CVF-toxiciteit, noch 'primary non-function' van de cavialever verantwoordelijk waren voor het vroege overlijden. Zowel syngene cavialever transplantatie als geïsoleerde leverperfusie bij de cavia resulteerde eveneens in vroege dood van de ontvangers. Daarom werd door ons geconcludeerd dat de cavialever ongeschikt is voor transplantatie vanwege zijn gevoeligheid voor chirurgisch trauma en ischemie/reperfusie schade.

In het cavia naar rat levertransplantatie model zou het verwijderen van de vrijgemaakte vasoactieve en immunoactieve stoffen minder snel kunnen gaan als gevolg van de gestoorde leverfunctie. In het harttransplantatie model in dezelfde species combinatie, waarbij een normale leverfunctie aanwezig is, leidt reperfusie van het harttransplantaat echter nog steeds tot vroege ontvangerdood tot in 40% van de gevallen. Er kan dus worden gesteld dat niet alleen leverfunctie als zodanig een belangrijke factor is, maar ook de hoeveelheid vasoactieve en immunoactieve stoffen, vrijgemaakt door het getransplanteerde caviaorgaan. Ergo, caviaorganen in het algemeen zijn gevoelig voor het chirurgisch trauma en ischemie/reperfusie schade geïnduceerd door transplantatie.

Omdat het onmogelijk was de veronderstelde immunologische bevoorrechte positie van de cavialever te onderzoeken, werd het onderzoek van dit fenomeen uitgevoerd in het "pseudo-discordante" hamster naar gesensitiseerde rat model. Wij toonden aan dat HAR in levertransplantatie bewerkstelligd werd door IgG, maar niet door IgM. Deze predominantie bestond niet voor harten. In normale concordante hamsterhart en -lever transplantatie zijn de overlevingstijden van levers significant langer dan die van harten. De opgewekte antilichaam respons is in eerste instantie van het IgM-type waarna het verandert in een IgG-type. Er bestaat geen verschil in immunogeniciteit tussen harten en

levers. In het cavia naar rat model zijn de voorgevormde natuurlijke antilichamen voornamelijk van het IgM-type. In dit model worden harten hyperacuut afgestoten, terwijl leverrecipiënten overlijden met niet afgestoten transplantaten. IgM deposities waren marginaal of afwezig. Op grond hiervan kwamen wij tot de conclusie dat zowel in concordante als in discordante xenotransplantatie, de lever een immunologisch bevoorrechte positie bekleed vergeleken met het hart. De lever, maar niet het hart, is minder gevoelig voor IgM-gemedieerde afstoting. Ook in de mens zijn de voorgevormde antilichamen voornamelijk van het IgM-type. De afwezigheid van (hyper-)acute afstoting na klinisch gebruik van de varkenslever bij extracorporele perfusie verklaart en ondersteunt eveneens de bevinding dat de xenogene lever minder gevoelig is voor IgM-gemedieerde afstoting.

Er kunnen geen harde conclusies worden getrokken wat betreft de verminderde gevoeligheid van xenogene levers voor antilichaamgemedieerde cellulaire afstoting. Het lijkt echter dat de lever niet immunologisch bevoorrecht is voor antilichaamgemedieerde cellulaire afstoting omdat het met dezelfde snelheid afstoot als het hart.

Fysiologische compatibiliteit van discordante cavialevers in ratontvangers kon niet worden onderzocht als gevolg van de afwezigheid van lange-termijn transplantaatoverleving. Vergelijking van veterinaire fysiologie vertoonde echter ernstige verschillen in haematologie en in elektrolyten en enzymatische concentraties. Vanuit de knaagdierfysiologie wordt gededuceerd dat cavia naar rat orgaantransplantatie niet zal resulteren in lange-termijn overleving door haemodynamische en biochemische incompatibiliteiten. Vergelijking van de beter gedocumenteerde varken- en mensfysiologie duidt op grote verschillen tussen beide species. Orgaantransplantatie van aan de mens nauw verwante species, zoals de baviaan en chimpansee, resulteerde reeds in fysiologische veranderingen. Deze bevindingen suggereren in hoge mate dat speciespecifieke fysiologie een belangrijke barrière is in xenotransplantatie.

Het is niet waarschijnlijk dat xenogene levertransplantatie in de klinische praktijk zal worden toegepast zonder additionele genetische modificatie om immunologische barrières te overwinnen. Tevens zal de aandacht in de toekomst gericht moeten worden op de fysiologische disbalans in leverfunctie tussen verschillende species, in het bijzonder tussen varken en mens. Hoewel de lever een immunologisch bevoorrecht orgaan is, geloven wij niet dat xenogene levertransplantatie in de nabije toekomst een reële optie zal zijn vanwege de specifieke beperkingen, zoals hierboven genoemd. Verder kan men zich voorstellen dat vanwege de progressie in kloneringtechnieken en ontwikkeling van artificiële organen, deze toepassingen xenotransplantatie van de lever voorbij zullen streven.

Addendum

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Nawoord en dankwoord

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Notes