Immunological aspects of clinical and experimental cardiac valve allograft implantation

Colofon

ISBN 90-9014983-X

Immunological aspects of clinical and experimental cardiac valve allograft implantation

This thesis was prepared at the Department of Cardio-thoracic Surgery and Internal Medicine, Erasmus University Rotterdam and University Hospital Rotterdam-Dijkzigt, The Netherlands.

Cover illustration: aortic valve allograft
Printed by Haveka B.V., Alblasserdam, The Netherlands

Immunological aspects of clinical and experimental cardiac valve allograft implantation

Immunologische aspekten van klinische en experimentele allogene hartklepimplantatie

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. dr. ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 14 november 2001 om 13.45 uur

door

Frans Bing Sien Oei

geboren te Fukien (China)

Promotiecommissie

Promotor:	Prof. Dr. A.J.J.C. Bogers
	Prof. Dr. W.Weimar
Overige leden:	Prof. Dr. F.H.J. Claas
	Prof. Dr. B. Mochtar
	Dr. R.L. Marquet

The studies presented in this thesis were in part supported by grant 96.177 from the Netherlands Heart Foundation.

Financial support by the Netherlands Heart Foundation for the publication of this thesis is atefully acknowledged.

~ Van de maan af gezien zijn wij allen even groot ~

Multatuli

Contents

CHAPTER 1

General Introduction

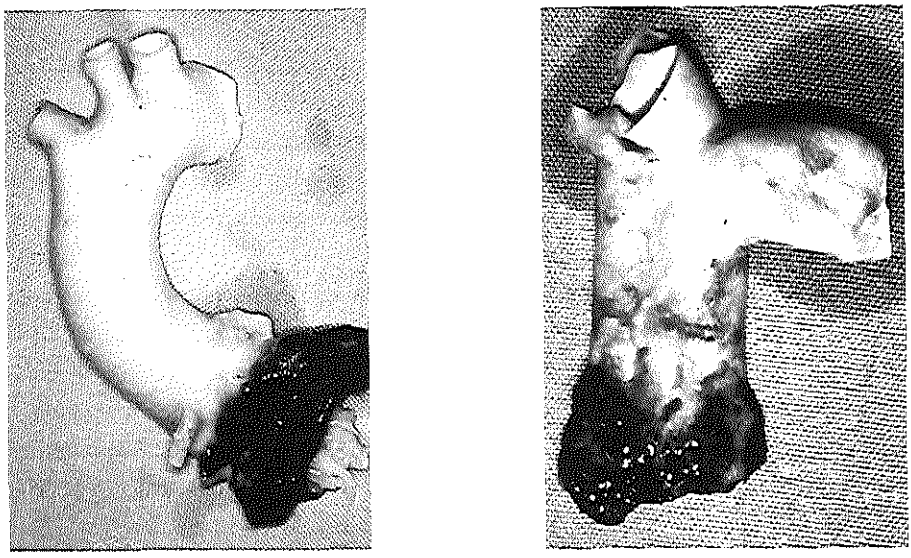
F.B.S. Oei

The history of cardiac valve allograft implantation

The documented history of allogeneic cardiac valve implantation began in 1952. In that year, the first successful implantation of an canine aortic valve in the descending aorta of another dog, was carried out by Conrad Lam *et al.* [1]. Driven by the succes seen in this animal model Murray *et al.* [2] started to insert fresh human aortic valves in the descending aorta of patients with severe aortic insufficiency in 1956. The function of these valves was reported to be satisfactory for up to 6 years [3]. After the introduction of open heart surgery around 1962, the replacement of aortic valve by inserting an aortic allograft into the subcoronary position was proposed by Duran *et al* [4] and completed with clinical success by Ross *et al.*[5]. In that same period, Barratt-Boyes *et al.* [6]from Auckland, New Zealand reported that they also started to insert aortic valve allografts in the subcoronary position independently from the London group.

As the primary clinical implantation of aortic valve allografts was performed for replacement of a diseased aortic valve, the use of these valves soon extended to other applications. In 1966, the use of aortic valve allografts in right ventricular outflow tract reconstruction (pulmonary valve position) was introduced [7]. With the description of this successful application by Ross and Somerville, the correction of many previously inoperable congenital cardiac defects soon became possible. Thereafter, Yacoub and his group proposed replacement of the mitral and tricuspid valve by inserting inverted, fresh unstented aortic valve allografts in 1969 [8]. After initially good results however, the long-term outcome appeared to be disappointing, a problem which is primarily related to the incidence of valve degeneration and endocarditis. In 1972, the replacement of the entire aortic root and valve by an aortic valve conduit was introduced in aortic valve replacement therapy for re-operations and infective endocarditis related aortic valve and aortic wall pathology [9].

The implantation of a pulmonary valve allograft in pulmonary valve position was first suggested by Eguchi *et al.* in 1968 [10] and two years later by Seki *et al* in laboratory experiments with dogs [11]. It was not until 1983, that the pulmonary valve allograft was clinically introduced as conduit for right ventricular outflow tract reconstruction by Ross *et al.*[12]. With regard to RVOT reconstruction, pulmonary valve allografts include better qualities regarding valve thickness, transvalvular gradient and handling. Additionally, several clinical follow-up studies have demonstrated that these pulmonary allografts are less prone to calcify after right-sided implantation when compared to aortic allograft conduits, thus gaining better long-term results. It is therefore that the pulmonary valve allograft is nowadays generally accepted as the conduit of choice for right ventricular outflow tract reconstruction especially in children with complex pulmonary anatomy [13]



Cryopreserved and thawed aortic valve allograft including the aortic root, ascenders and arch (left). Cryopreserved and thawed pulmonary valve allograft including the truncus and left and right pulmonary artery (right)

Clinical applicability of mitral and tricuspid valve allografts has already been suggested in the early years of valve allograft implantation by Robicsek *et al.* [14], but the concept has never reached clinical acceptance mainly due to technical failures based on early chordal rupture. However, in the past five years the principle of using a mitral or tricuspid allograft to replace a diseased mitral or tricuspid valve seems to regain the interest of some cardiac surgeons [15, 16].

After the initial appreciation during the first decade the aortic valve allograft slowly lost popularity in the second decade due to two important development in valve replacement surgery. Previously, aortic valve allografts were inserted freshly and directly after harvest [17]. As a consequence, the growing popularity and the lack of competition from other biological valves created a dramatic issue concerning allograft availability. In order to solve the problem of limited donor availability various preservation methods like fresh wet storage at 4°C or freeze-drying has been employed. Additional concern regarding transmission of infection led to the development of different aggressive sterilization protocols including incubation in highly concentrated antibiotics, glutaraldehyde pretreatment or irradiation [6, 18, 19]. Although these protocols did reduce the possibility of bacterial transmission and increased the storage time and tissue availability, the clinical durability appeared to be drastically decreased compared to fresh untreated allografts [17]. Secondly, in the mid-1970s, newly developed reliable biological prostheses became available.

Besides good initial reliability and unlimited availability, these bio-prostheses include less demanding implantation techniques when compared to human tissue valves. Based on these advantages the bio-prostheses became increasingly popular in the period after 1976.

During the late seventies en early eighties, research regarding valve allograft viability has been intiated at the Thoraxcenter in Rotterdam (van der Kamp *et al* and Mochtar *et al*). Because long-term behaviour of valve allograft was supposed to be dependent on the maintenance of its extracellular matrix., preservation protocols were studied in order to increase graft viability. Especially the behaviour of matrix fibroblast was analyzed as these cells were regarded as the most important cells for maintenance of the extracellular matrix [19b].

Reappraisal of donor valve allografts came in the third decade as improved sterilization methods and adequate procurement protocols had prolonged the allograft durability [20]. The introduction of alternative preservation methods like cryopreservation and storage in liquid nitrogen described by O'Brien and Angell has extended the availability of tissue grafts [21, 22]. The possibility of long-term storage and the ever-increasing demand of valve allografts stimulated the foundation of regional heart valve banks, which further improved the availability of these allografts. Sixty-eight heart valve banks are registered over the world in 1995 and the numbers are still growing [23].

In the Netherlands, cryopreserved aortic and pulmonary valve allografts are prepared, processed and stored at the Heart Valve Bank Rotterdam, which was established in 1988. The valve bank is located in the Thoraxcenter of the Erasmus Medical Center in Rotterdam. Valves are obtained from heart beating donors or non-heart beating donors. Heart beating donors consist of donor hearts obtained from multi-organ donation procedure, which by any reason was not suitable for transplantation. A second heart beating donor source is the recipients' "domino"-heart that becomes available after a heart transplant procedure. Non-heart beating donors which represents the largest source for valve allografts are post-mortem donors in which the donation procedure has been carried out within 24 hours after circulatory arrest. The acceptance of a heart valve donor is submitted to strict criteria, including absolute and relative contra-indications [24]. Regulation of donor acceptance, procurement, transport and allograft allocation is executed by Bio Implant Services (BIS), a non-profit institution related to the Eurotransplant foundation. After aseptic explantation of the donor heart by a surgical team of BIS, the complete heart is transported to the Heart Valve Bank Rotterdam. Preparation of the aortic and pulmonary valves under sterile conditions is followed by incubation of the allografts in a low-dose antibiotic solution for 6 hours at 37°C. Cryopreservation is carried out by controlled rate freezing (±1°C/min) until -80 °C and stored in the vapor phase of liquid nitrogen (-150 °C) waiting for implantation [25]. To prevent structural and cellular damage the allografts are cryopreserved in storage medium including dimethylsulfoxide (DMSO). The allografts are thawed shortly before implantation according to the standard protocol of the Heart Valve Bank [25].

At this moment the Heart Valve Bank Rotterdam also processes and stores cryopreserved aortic or pulmonary artery tissue patches and thoracic aortic allografts as valuable clinical tissue implants [26]. By the introduction of a European network of co-operating heart valve banks in e.g. Germany, Spain, Sweden and the UK, an international allocation system, which is operated by BIS, could be implemented in order to enhance the availability of cryopreserved valve allografts.

Clinical perspectives of cardiac valve allograft implantation

Implantation of valve prosthesis improves physical condition and life expectancy of patients with acquired or congenital cardiac valve diseases. Different types of prostheses are available nowadays, with specific advantages and disadvantages. Mechanical valves have excellent durability but include high risk of thromboembolic complications that necessitates lifelong anticoagulation [27]. Therefore, the use of mechanical valves is relatively contraindicated among patients with an active life style or a wish for pregnancy. Both human and xenograft (porcine or bovine) valve-prostheses lack thromboembolic complications and therefore are implanted without the need for routine anticoagulation [28]. However the major limitation of xenografts is the progressive structural failure, which is more often seen in younger patients [29, 30]. Human tissue valve grafts, on the other hand, include greater durability and demonstrate better resistance to infective endocarditis [31].
Human tissue valve prostheses have been introduced as "homografts", defined as valve from an individual of equal species. However, in transplantation nomenclature intra-species organ or tissue grafts are generally referred as "allografts". Using this term, a differentiation between tissues obtained from the patient self (autograft) or from other individual of the same species (allograft) could be made. In this thesis a valve allograft is defined as a valve prostheses obtained from another individual of the same species.

Reports concerning the clinical outcome of valve allograft implantation in the right or left ventricular outflow tract have been inconsistent. The variability in surgical techniques, valve preservation methods, patient age and various definitions of structural valve failure led to inconsistency in reports and made valid comparison between different follow-up studies very difficult or even impossible. In order to uniform clinical studies regarding valve surgery Edmunds *et al.* introduced guidelines to report clinical morbidity and mortality after valve surgery [32]. As stated in the guidelines, failure of the valve prostheses should strictly be defined as valve failure when surgical removal of the valve (re-operation) is necessary or when death of the patient occurs. However, this robustly black-or-white end-points could lack sensitivity as clinical indications for valve deterioration are not taken into account and consequently underestimates the actual incidence

of valve failure. Therefore, is has been proposed to generally adopt the term valve *dysfunction* to report clinical signs and symptoms that reveal abnormal function of the valve.

Valve allograft deterioration resulting from intrinsic structural graft pathology defined as structural valve failure (e.g. valve stenosis, calcification, leaflet tear) has been frequently described. The mechanisms of valve allograft failure are interrelated and include tissue degeneration and geometric distortion that are influenced by multiple risk factors.
These factors can be generally divided in three relating categories: patient characteristics, valve (donor) characteristics and implantation techniques [33].

Long-term follow-up studies regarding implantation of valve allografts in aortic position revealed good clinical results, especially in adults. Actuarial freedom from valve related re-operation, which is regarded as an objective indicator for structural valve allograft failure varied in these studies between 75-89% in 8 years [34-36]. Our center recently reported a comparable result of aortic root replacement indicating a freedom of valve related re-operation of 82% at 8 years [37]. Some studies reported an incidence of allograft degeneration in aortic position at a disappointing rate, especially in young recipients [38-40]. Implantation of valve allografts in the pulmonary circulation is performed for reconstruction of the right ventricular outflow tract in patients with complex congenital heart defects or in an pulmonary autograft procedure for aortic valve disease. Reports concerning the clinical outcome of right sided implantation of valve allografts reveal more structural degeneration when compared to allograft implantation in the aortic position, especially in the infant population with complex congenital defects. Overall freedom from valve related re-operation were between 80 to 90% at 5 years [41-44]. In infants younger than three months old, the results that have been reported are even worse, as the freedom for re-operation at 5 years have declined to only 22% [45].

Because implantation of aortic or pulmonary valve allografts are performed without matching of tissue antigens like blood group (ABO) and Human Leucocyte Antigens (HLA), the human equivalent of the Major Histocompatibility Complex (MHC) antigens, immune activation of the recipients has been expected. Since the introduction of clinical implantation of valve allografts, tissue immunogenicity has been a matter of debate [46-48]. The fact that relatively good medium-term clinical results are achieved without matching of donor and recipient tissue antigens or without the use of immunosuppression, these valve allografts has been regarded as tissue with low immunogenicity. Nevertheless, accumulating evidences for immunological associated valve deterioration has been provided by past and recent experimental animal and patient related studies.

Principles of immunological responses in tissue and organ transplantation

The immune system

Development of the immune system during the evolution has been driven primarily by the need to eliminate infectious agents and protect against re-infection. As a results, the human immune system could be roughly divided into an "innate" or non-specific and a "memory" specific immune system. The "innate" or non-specific immune system consists of cellular components (e.g. macrophages, neutrophils and natural killer cells) and soluble non-cellular components (e.g. lysozymes, complement and acute-phase proteins). It is effective against certain antigeneic stimuli by quickly eliminating the antigens by phagocytosis. Limitation of this system is the lack of "memory" needed during re-infection and the limited variety of the response. Therefore, a superposed, specific or adaptive response had evolved to meet the continuous evolutionary antigeneic challenge. Both B- and T-lymphocytes are important effector cells in this specific system. Activated B-lymphocytes (=plasma cells) produce soluble antibodies able to bind to specific pathogens and increase the efficiency of killing and/or phagocytosis of the pathogen bearing cells. The T lymphocyte population can be divided in helper (HTL) and cytotoxic (CTL) T lymphocytes. Helper T lymphocytes (HTL) regulate the specific immune system by interaction with cytotoxic T lymphocytes (CTL), B-cells and macrophages through the release of soluble mediators called cytokines. CTL are important effector cells that are able to kill infected host cells or foreign (donor) cells by direct contact and secretion of perforins and granzymes. The efficiency of the specific immune system is based on the principle of clonal expansion and induction of surveying "memory" T and B cells in a large heterogeneous T and B cell population [49].

In physiological situation the immune system protects us from lethal infections, but in organ or tissue transplantation, the same system is preventing transplant recipients from graft acceptance.

The T-cell receptor

The ability of T- and B-lymphocytes to recognize foreign structures (antigens) is mediated by antigen-specific receptors on the surface of these cells. Immunoglobulin (Ig) molecules are the antigen specific receptors on B-lymphocytes, while T lymphocytes have the T cell receptor (TCR) for this function. Recognition of foreign antigens by T lymphocytes depends on the possible interaction between the T cell receptor (TCR) and the antigen itself captured in a Major Histo Compatibilty (MHC) molecule of an antigen-presenting cell. This complicated antigen recognition known as the MHC restricted recognition arose during evolution for effective elimination of extra-cellular pathogens and virus infected autologous cells.

The T- cell receptor (TCR), a transmembrane protein closely linked to the CD3 complex, which plays an important role in signal transduction from the cell surface (TCR) to cytoplasm [50]. The main T cell population that mediates the specific immune response when encountering antigens,

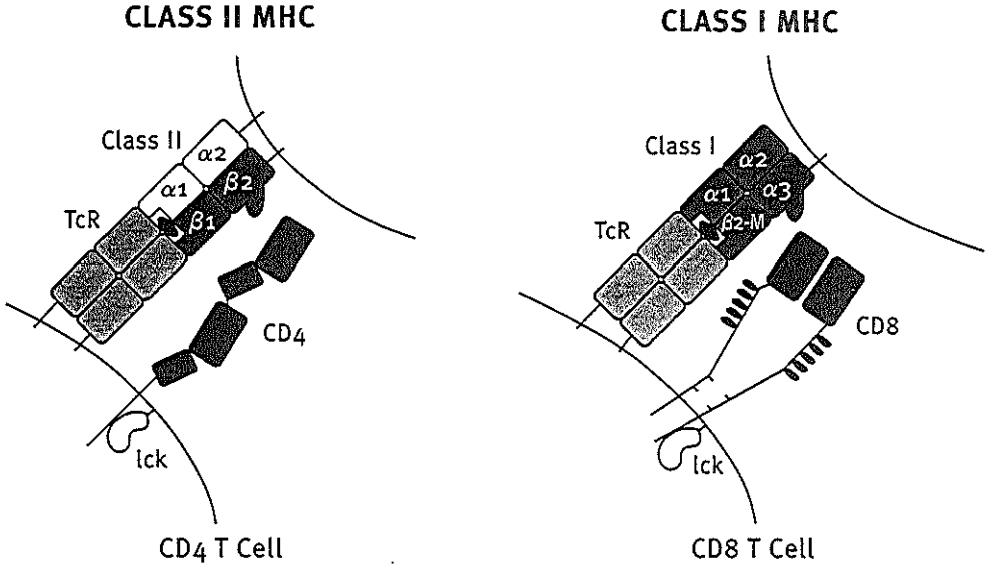
have TCR's consisting of α and β chain each with variable (V) and constant (C) domains [51]. The heterogenicity of the T cell population (the T cell repertoire) is based on the diversity in the variable (V) domains of the TCR which consist of 3 hypervariable regions. The first two regions (CDR 1 and 2) form the outer regions of the TCR, while the third (CDR3) region forms the central part. It has been suggested that during antigen presentation the outer regions (CDR1 and CDR2) interact with the MHC molecule of the antigen presenting cell and the inner region (CDR3) binds to the antigen captured in the groove of the antigen presenting MHC molecule [51]. The normal T-cell repertoire contains a high frequency of T-cells capable of responding to allogeneic MHC molecules [52].

The accessory molecules CD4 and CD8

The introduction of methods to distinguish leukocyte subpopulations by using antibodies directed against specific surface glycoproteins, more insight regarding the mechanisms of cellular immune response was achieved. To identity different cell markers these surface glycoproteins are given numbers according to the cluster of differentiation (CD). Initially, the helper T lymphocytes (HTL) became known as CD4 positive T cells and the cytotoxic T lymphocytes (CTL) as $CD8^+$ T-cells [53]. CD8 and CD4 molecules on the T-lymphocyte surfaces are accessory molecules that stabilize TCR-to-antigen interaction by binding to the non-polymorphic region of HLA class I and Class II, respectively [54,55]. Simultaneous binding of TCR and CD4 or CD8 to the same MHC/peptide antigen complex is required for maximal T cell stimulation [56,57]. The importance of these accessory interactions for T cell activation and function is highly depending on the affinity of the TCR-to-MHC interaction or the density of TCR and MHC/peptides present on the responding and target cell surfaces, respectively. With other words, the necessity of T cells to become activated or functional with or without the accessory binding of CD4 or CD8 is indicative for the avidity of the T-cell [58-60]. By using CD4 or CD8 specific antibodies during in vitro tests, sub-populations of T cells with high or low avidity for antigens could be identified [61,62]. In the effector phase, the cytotoxic function of $CD4^+$ or $CD8^+$ CTL with low avidity are inhibited by the CD4 or CD8 antibody respectively, whereas CTL with high avidity are not inhibited.

The specific T-cell recognition of an antigen by the TCR is known as the "first signal", which is on its own not sufficient to activate the T-cells to respond to the presented antigen. In addition to the "first signal", a co-stimulatory signal involved as a "secondary" signal has been demonstrated to play a key role in T-cell activation. The co-stimulatory signal provided by the interaction of CD28 on the responding T cells and CD80 and CD86 on the antigen presenting cells loaded with the antigen is obligatory for effective activation of T-cells [60,63]. Additionally, interactions of other cell surface molecules (e.g. CD2-LFA3, ICAM1-LFA1) provide stable physical cell to cell adherence which is required during signal transduction between stimulatory cells and responding T cells. Furthermore, in addition to these adhesive and signaling interactions, a third signal is involved in the initiation

phase of an effective immune response. This third signal is delivered by several cytokines, which are produced by both antigen presenting cells and responding T cells.



Schematic presentation of the CD8 and CD4 molecules, interacting with MHC class I and class II

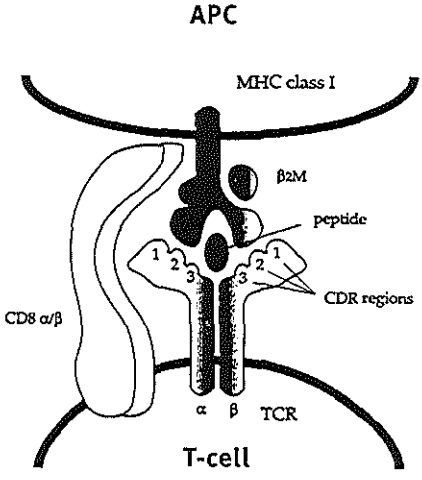
The Major Histocompatibility Complex (MHC)

In contrast to B lymphocytes which recognize antigens directly via binding with their immunoglobulins, the activation of T cells depends on an additional structure: the Major Histocompatibility Complex (MHC) molecule, also in human known as the Human Leukocyte Antigen (HLA). The fact that T cells only recognize antigens in the context of MHC molecules is known as the MHC restriction. Additionally, allogeneic MHC can directly induce an immune response when present on dendritic cells of donor origin. Therefore, the MHC molecules are regarded as one of the most important structures involved in allograft rejection. Consequently, by matching donor and recipient histocompatibility antigens rejection of the allograft can be avoided.

The highly polymorphic Human Leukocyte Antigens (HLA) are encoded by a set of genes, located on chromosome 6 [64]. Two sets of HLA molecules are identified to be important in activation of the specific immune system which is associated with allograft rejection. The HLA class I molecules are present on all nucleated cells of the body. On the other hand, HLA class II molecules have a more restricted distribution as they are primarily found on cells involved in antigen presentation (dendritic cells, macrophages, monocytes, B-cells) and on activated cells such as activated T lymphocytes and epithelial cells and endothelial cells).

The high degree of polymorphism of the HLA-system results in large numbers of HLA-specificities [65,66]. HLA class I molecules are surface glycoproteins and consist of a highly polymorphic heavy chain (α-chain) and is non-covalently associated with a β2-microglobulin; a non MHC-encoded protein. The α-chain is encoded by the HLA-A, -B and -C genes of the HLA complex, which results in extreme polymorphism. The largest (extracellular) part of the heavy chain is organized into three globular domains (α1, α2, α3) that are involved in alloantigen recognition. The α1 and α2 domains are the most polymorphic domains of the class I genes [69]. Within the groove formed by these two domains, peptides of 8 to 10 amino-acids are bound as is required for presenting antigens to T-cells.[70]. Several polymorphic residues are located in the part of the molecule that constitute this groove, resulting in the ability to bind a diversity of peptides.

HLA class II molecules, encoded by class II genes HLA-DR, HLA-DQ and HLA-DP are heterodimers consist of an α and β-polypeptide chain, each with two polymorphic extracellular domains (α1 and α2; β1 and β2), a transmembrane and a cytoplasmic region. The membrane distal α1 and β1 domains are highly polymorhic while the membrane proximal α2 and β2 domain are more conserved [70]. Analogous to HLA class I molecules, the polymorphic α1 and β1 domains form the peptide binding side, while the groove of class II molecule is open at both ends resulting in binding of longer peptides [71].



Schematic presentation of the key elements of the T-cell receptor (TCR) for recognition of peptides presented by HLA molecules. These elements are named CD1, CD2, CD3 here marked as 1, 2 and 3. Depicted is a CD8 T-cell which recognizes an HLA Class I molecule on a antigen presenting cell (APC).

After synthesis in the cell, both HLA class I and class II molecules are transported through complex intracellular routes and bind peptide at different moments during their courses to the cell surface. This phenomenon results in a dichotomy of antigen presentation to T cells [73]. HLA class I

molecules present antigens predominantly of intracellular origin (endogeneous antigens) and class II molecules present mainly antigens of extracellular origin (exogenous antigens). Analogous to this dichotomy in antigen presentation, there is also a duality in the T cells that reacts with antigens presented by either HLA class I or II molecules. As the accessory molecules CD8 and CD4 bind to α3 and β2 domains respectively, CD8 bearing T cells recognizes (endogenous) antigens presented by HLA class I while CD4 positive T cells recognize (exogeneous) antigens associated with HLA class II molecules [74,75].
Allo-recognition of foreign tissue including foreign MHC is more complex and can be either direct (MHC on donor (allo) cells with or without allo-or self-peptides) or indirect (donor (allo) peptides presented by autologous MHC on recipients own antigen presenting cells (APCs)) [76].

Antigen-presentation pathways
Transplantation of viable allogeneic tissue or organs creates an unique immunological situation where two sets of antigen presenting cells (APC) are available for stimulation of the immune system. Recipient T cells can recognize donor (allogeneic) MHC antigens by two different pathways, either directly as intact molecules on donor derived APCs or indirectly as peptides in the groove of host (autologous) MHC molecules after antigen processing by autologous APCs.

Direct antigen presentation (fig.4a) is characterized by direct engagement and response of recipient T-cells to intact allogeneic (donor) MHC molecules. Direct engagement of non-self (allogeneic) MHC molecules is in combination with a non-self (allo)-peptide or a self-peptide on the surface of allogeneic antigen-presenting cells (APC). Direct T-cell activation is possible via two kinds of APCs: tissue specific (professional) APCs (e.g. Langerhans' cells in the skin, Kupffer cells in the liver and dendritic cells in the heart and kidney) or cells with APC-function (activated endothelial cells, macrophages, B-cells)[49]. The response of T cells to allogenic MHC molecules is more vigorous compared to "normal" enviromental antigens which could be caused by the powerful stimulation of T cells by allo-antigens expressed on allogeneic APC [77]. T cell responses that result in early, acute rejection seem primarily due to direct recognition of HLA allo-antigens present on donor derived dendritic cells (DC). Batchelor and colleagues gave evidence to this theory by demonstrating that mature allogeneic DC can induce acute graft rejection [78].

Indirect antigen-presentation (fig 4b) is characterized by T-cell recognition of antigens as processed peptides presented by self-MHC molecules. The common route for nominal antigen presentation is the indirect pathway in which self (autologous) APCs (DC and macrophages) capture and process allo-antigen and migrate to the secondary lymphoid organs where they trigger naive T cells [76]. Processing of allo-antigens is proceeded by internalization of the foreign antigens followed by degradation into peptides and incorporate into the cleft of MHC class I and II for presentation to T

cells [79,80]. Therefore soluble allo-antigens shed by graft tissue or cellular debris also are captured and processed by recipients DC or macrophages, similar to the mechanism of nominal antigen presentation [80]. Various studies have outlined the involvement of different haematopoetic cells in stimulation of the indirect response. Dendritic cells, monocytes and also activated T-cells or B-cells are able to have this function [82-85].
Although the existence of indirect and direct pathways of antigen presentation has been demonstrated in various experimental studies, their contribution in graft rejection is less well defined. The general consensus is that early after allogeneic organ or tissue transplantation, $CD4^+$ and $CD8^+$ T-cells of the host become activated via direct recognition of donor DC inside the graft. In time after transplantation, all donor DC will be migrated out of the graft and then are replaced by recipients APCs which activate $CD4^+$ and $CD8^+$T-cells by indirect recognition. As graft rejection is frequently experienced early after transplantation, it has been suggested that graft rejection is only triggered by direct presentation of MHC molecules to T-cells. However, additional observations suggest that indirect allo-recognition may enhance the rejection response and therefore plays a more significant role than expected [86].

Allograft rejection
Four types of graft rejection can be determined based on the specific time of occurrence. **Hyper acute rejection** appears within 24 hours after transplantation and is induced by pre-existing anti-donor antibodies [87]. The introduction of pre-transplant crossmatching reduces the incidence of hyperacute rejection. **Accelerated acute rejection** begins within 5 days after transplantation which pathogenesis is unclear but may involve anti-endothelial antibodies. **Acute rejection,** seen within the first year after transplantation may be due to cellular effector mechanism. Acute rejection is characterized by graft (organ) dysfunction and dense intersitial and perivascular infiltration of mononuclear cells with cellular damage and interstitial edema [88]. **Chronic rejection** occurs late (>1y) after transplantation and involves gradual loss of graft function and narrowing of graft arteries and microvasculature due to diffuse concentric intimal thickening. Proliferation of medial derived smooth muscle cells and fibroblast in the vascular intima may cause the intimal thickening [89]. However, the pathgenesis, which is probably multifactorial is not clearly understood yet.

The involvement of lymphoid cells (lymphocytes and macrophages) in acute graft rejection has been outlined already in the early years of transplant immunology by Medawar and other researchers [90]. Based on studies performed in the sixties it has been suggested for many years that cytotoxic T lymphocytes (CTL) are the principal effector cells in graft rejection [91]. Billingham proposed an alternative concept for graft rejection as he suggested acute rejection as an type of Delayed Type Hypersensitivity (DTH) mediated by Helper T cells (HTL). This concept became popular in the eighties because evidences of interleukin-2 (IL-2) associated graft rejection became available [92].

However, recent studies have demonstrated that the absolute functional separation of CD8+T-cells as CTL and CD4+ T-cells as HTL has become invalid. Experiments have shown that CD4+T-cells are also able to kill MHC class II+ target cells while CD8+T-cells also can function as HTL by producing cytokines [93]. The concept of MHC class I and II restriction for CD8+ and CD4+ T-cells respectively however remains valid as both cells are activated by autolgous (self) MHC class I and II respectively presenting processed allo-antigens [94]. There is ample evidence that both T cell populations are important in graft rejection and may very well complement each other to achieve maximal effectiveness of the immune system.

Monitoring of T-lymphocytes frequencies after clinical transplantation.

After allogeneic tissue or organ transplantation there is inevitably the risk of graft rejection, even in HLA well-matched recipients with adequate immunosuppression. Besides clinical signs of graft rejection, diagnosis of rejection can only be confirmed by histological evaluation of biopsies taken from the graft. As the biopsy procedure is an invasive method, there is always a risk of complication which is often inconvenient for the patient. Many attempts have been undertaken to find less invasive ways to monitor graft rejection including various in vitro techniques. As cytotoxic (CTL) and helper T (HTL) lymphocytes play an important role in rejection, monitoring of these cells in peripheral blood could in theory be indicative for acute graft rejection.

The theory of the cytotoxic T lymphocyte (CTL) as an important mediator of allograft rejection has been stated by Govaerts and was supported by many experimental studies [95]. The cell-mediated lympholysis (CML) assay, developed by Brunner was the most frequently used in vitro assay to detect CTL allo-reactivity [96]. However, quantification of the allo-reactivity remained the major issue. To overcome this problem a sensitive and reproducible "Limiting Dilution Assay" (LDA) was introduced [97]. Applying this technique in humans, it was found that each individual has a different baseline precursor CTL (CTLp) frequency specific against different HLA class I and II allo-antigens [52, 99]. Additionally, it was found that the higher the CTLp frequency the more potent the immune response will be that takes place after the antigenic challenge. Enumeration of CTLp in peripheral blood of different transplant recipient revealed increase in CTLp frequencies during rejection of bone marrow, cornea, kidney, heart and liver transplants [100,101]. However, these studies also reported frequency increase in patients without any signs of rejection and vice versa [102]. Therefore the clinical relevance of this conventional assay remains insufficient. In this respect, transplant immunologists have suggested that a change in quality (e.g. naïve or activated, low or high avidity) instead of the quantity of CTL populations is associated with the rejection process. Conventional LDA only detects the total frequency of CTLp against certain allo-antigens and not the quality (e.g. receptor (TCR) avidity and co-stimulatory molecules expression) of the allo-antigen specific CTLp. By using monoclonal antibodies directed against the accessory molecules CD8 or CD4 (stabilizing molecules for TCR-antigen interaction) it is possible to distinguish low avidity cells from high avidity

cells. A low avidity CTLp is not able to lyse the target cell, during the effector phase, when their accessory molecules are blocked by monoclonal antibodies. In the clinical setting, the number of high avidity CTLp specific for donor HLA class I antigens appears to be higher in cardiac transplant recipients experiencing rejection as compared to other non rejecting recipients [103]. An increase of the numbers of CTLp could be observed in peripheral blood and in the graft itself during acute rejection [104,105].
The proliferation of most CTLp depends on the presence of interleukin-2 (IL2) [106]. Therefore, enumeration of the frequencies of IL2 producing Helper T lymphocytes (HTL) provides additional information in different transplantation settings [107]. Promising reports have been published regarding the predictive value of HTL frequency analysis in peripheral blood for the occurrence of rejection in bone marrow or cardiac transplantation [108,109]. Vaessen *et al* and Debruyne *et al* have demonstrated that an increase of HTL frequencies in peripheral blood is correlated with acute rejection of respectively heart or renal transplants [110].

Immunogenicity of allogeneic valves

Valve allograft immunogenicity: Animal studies

Since the introduction of human cardiac valve allografts as the ultimate biological valve prostheses, the immunogenicity of the valve grafts has been a matter of debate. Studies involving experimental animals have demonstrated the ability of cardiac valvular tissue to sensitize allograft recipients [111-113]. In canine and bovine models, sensitization of valve recipients was demonstrated by a more rapid rejection of subsequent skin grafts from the same donor. These studies also provided histological evidence of valve tissue rejection, which was associated with (allogeneic) activation of the immune system. However structural deterioration of valvular tissue did not results in dysfunction of the valve grafts. Since these studies were performed in a Major Histo Compatibility (MHC) undefined model and the use of a composite valve graft, their results could be misleading.
By using inbred rat strains with well defined MHC specificities, immunological studies regarding valve allograft transplantation could be performed more accurately. Heslop and colleagues demonstrated in MHC incompatible rat strains the differences in immunogenicity of various valve allografts components [114]. They suggested that the degree of the immune response is related to the degree of histo-incompatibility between donor and recipient. Their theory has been confirmed by Gonzalez-Lavin using genetically related and unrelated dogs [115]. Thiede et al examined in a comparable rodent model the relation between antigeneic load and the level of sensitization by intravascular implantation of various amounts of allogeneic valve leaflets. By using skin graft rejection time as a marker for sensitization they could not demonstrate differences in the degree of sensitization [116]. Their results indicate that only the degree of histo-incompatibility but not the antigeneic load plays a role during induction of the allogeneic response. Lupinetti and colleagues

used rat models to study the association between histo-incompatibility and endothelial cell replication and also valve allograft calcification [117-119]. Other in vivo studies also showed strong relationships between immune stimulation due to allogeneic implantation and structural destruction of valvular tissues [120-123]. In these studies, histological evidences of both acute and chronic rejection were observed generally. However tissue deterioration was never correlated with valve function because analysis of valve competence of valve transplants was not feasible due to less appropriate implantation techniques [124].
In earlier studies the time of additional skin graft rejection was used as a measurement of immune reactivity induced by allogeneic valve transplantations. This method is complicated and is liable to subjective interpretation. Therefore, it may not be accurate enough to monitor the kinetics of the allogeneic immune response. Zhao et al demonstrated by using inbred rat strains and heterotopic valve implantation, that monitoring of immune competent cells could reflect the immunological state of recipient animals [125]. The authors observed an increase of donor-specific cytotoxic T-lymphocyte frequencies within the spleen of aortic valve allograft recipient, which is accompanied by an increase in the level of anti-donor antibodies. Similar to studies regarding organ transplantation, certain donor-specific cellular reactivity appeared to correspond with histological rejection of the valvular tissue [122].

Valve allograft immunogenicity: Patient related studies

Blood group antigens (ABO) and Human Leucocyte Antigens (HLA) are regarded as antigens which are able to provoke a strong immune response in antigen incompatible organ or tissue transplantation. The activated immune system induces various degrees of tissue injury. Depending on the severity of tissue injury combined with the occurrence of graft dysfunction, the diagnosis of graft rejection is made. As ABO and HLA tissue antigens are present on cells in viable valve allografts, they could be involved in structural valve deterioration. The importance of ABO antigens matching has been recommended by authors in different experimental studies [126-127]. However, the relationship between ABO antigen incompatibility and clinical valve allograft failure could not be confirmed by clinical studies [48, 128-130]. Therefore, consecutive studies have been focussed on other antigens present on the cell surface: the Human Leucocyte Antigens.

In vitro studies have demonstrated that cultured valvular fibroblasts and endothelial cells were able to express HLA class I and II antigens and were able to stimulate lymphocytes in vitro [131,132]. More importantly, cultured class II positive endothelial cells could induce direct stimulation of resting and memory CD4+ T lymphocytes [133,134]. For this reason and also because endothelial cells form the primary barrier between the blood flow and valvular tissue, the viability of endothelial cells and fibroblast has been considered, to be an important factor contributing to immune stimulation. However, only one single histological study scarcely described the presence and

localization of endothelial cells and more interestingly the presence of HLA-antigens in human aortic valves by immunohistochemical staining. Yacoub and colleagues demonstrated the presence of HLA class I molecules on endothelial cells and cells present in the valvular matrix, but found no HLA class II expression on endothelial cells [135]. HLA class I antigens gradually disappeared after prolonged sterilization and storage (within 48 h). Interestingly, class II positive cells were only present in the sub-endothelial layer. They suggested that these cells are leukocytes or dendritic cells. Dendritic cells are professional antigen presenting cells, which express different surface markers including complement receptors, adhesion molecules, HLA class I and II antigens and most importantly the expression of CD80/CD86 accessory molecules. These surface molecules are necessary for stimulation of naive T cells, in order to induce an allogeneic immune response [76]. Because fibroblasts and smooth muscle cells could have polygonal or spindle-like appearances similar to dendritic cells (DC), the morphological identification of DCs in valvular tissue is difficult. Based on the expression of various surface antigens, immunohistochemical staining of valve section with specific antibodies could suggest the presence of DCs, however the presence of various cell populations in normal valvular tissue which express the same surface markers may interfere proper interpretation. Bobryshev and colleagues introduced nerve tissue protein S-100 staining as a specific marker to identify dendritic cells in the intimal layer of arteries. As this layer of the artery wall is not innervated and therefore do not contain nerve tissue, cells that are positive stained by S-100 antibodies should be regarded as dendritic cells [136]. Especially when fibroblasts and other mononuclear cells do not express S-100 surface proteins [137,138]. Based on these results Hogan and colleagues analysed aortic valve tissue for the presence of S-100 positive cells. They found a large amounts of spindle-shaped, polygonal S-100 positive cells in the matrix of the valve which they defined as dendritic cells. The amount of these cells appeared not to diminish by cryopreservation and could therefore be responsible for the immunogenicity of clinically used human aortic valve grafts [139].

In vitro immunogenicity of human valvular tissue has been analysed by Hoekstra and colleagues. They found that fresh valve pieces were able to stimulate HLA class I and II disparate peripheral blood mononuclear cells in vitro. After cryopreservation of valve tissue a significant decrease of stimulation was observed, which may indicate reduced viability of the cellular components in the cryopreserved valve [134]. However, in additional studies the same group found anti donor antibody in peripheral blood plasma of patients who received a cryopreserved valve allograft. The antibodies were specific directed against donor HLA class I and II antigens and therefore indicate that sufficient cellular components, baring HLA molecules, are left for immune activation [140,141]. The increase of circulating donor specific antibodies were also reported by other researchers performing comparable prospective studies [142-144]. Their observations all show an increase of anti-donor HLA antibodies four weeks after valve allograft implantation, which were still detectable one year after implantation.

In order to demonstrate cellular immune activation, Fischlein and Schutz introduced cytoimmunological monitoring (CIM) of peripheral blood from valve allograft recipients [145]. They reported an early (within 5 days) increase in the fraction of activated lymphocytes based on morphological appearances of circulating lymphocytes. The increase was more obvious in blood group (ABO) mismatch donor-recipient combinations when compared to matched combinations. Interestingly, the increase of activated lymphocyte fraction was spontaneously followed by a rapid disappearance of activated cells (after 7 days) in both groups. However, CIM appeared to be a non-specific method to diagnose graft rejection in cardiac allograft recipients [146]. Because, CIM is based on cell morphology and not on cell activity it may suffer from non-specific immunological reactivity (e.g. surgery, infection). By the use of mixed lymphocyte culture, Hogan and colleagues have shown a donor-specific immune response present in peripheral blood of cryopreserved valve allograft recipients. They found an increased cellular reactivity 30 days after valve implantation, which remained increased 3 months after implantation [143]. Although mixed lymphocyte cultures are useful for analysis of the donor-specificity of the immune response, it lacks sensitivity for detecting graft rejection.

Examination of allografts, explanted at re-operations or autopsy for structural valve failure, revealed the presence of specific immunoglobulins and activated complement (C3C) on the valvular surface [127,147]. Histo-pathological analysis of these allograft explants on the other hand demonstrated increasing loss of interstitial cells which correlates with the period of implantation [148-151]. Interestingly, several research groups have reported inflammatory cells, mainly T-lymphocytes, in valve allografts which were explanted shortly after the initial implantation, suggesting an immune mediated tissue degeneration. Hoekstra and colleagues were able to culture CD8 positive T lymphocytes from valve explants additionally exhibiting donor-specific cytotoxicity [152]. Despite evidences of increased humoral and cellular reactivity triggered by valve allograft implantation and indirect conformations of immune mediated tissue deterioration, direct association between anti-donor immune activation and structural valve failure is still elusive. The disability for histological monitoring of tissue samples in a clinical setting, similar to cardiac transplantation, forms the main obstacle for diagnosis of valve allograft rejection. For this reason, animal experiments have been performed to study immune related tissue injury more structurally. Since the specificity of tissue antigens (MHC) among experimental rodents are more defined and homologous, especially within inbred populations, they are often used for in vivo transplantation studies. Because orthotopic implantation of valve allografts involves cardioplegia it is not applicable for small animal studies. Implantation of aortic valve allografts in a heterotopic segment include several non-immunogeneic factors which may interfere objective histological assessment of the graft. For this reason, in vivo studies regarding the immune related valve allograft destruction and dysfunction must be performed in an immunological and hemodynamic suitable rodent model.

Aim of the thesis

The existence of an extensive and adaptive immune system as a sophisticated defense mechanism is needed by higher organisms including humans to prevent and overcome invasion of environmental pathogens. In tissue or organ transplantation however, activation of this immune system, by allogeneic tissue antigens, hinders the acceptance of the allogeneic graft.
The fact that implantation of human heart valve allografts is not proceeded by matching of tissue antigens like blood group antigens (ABO) or Human Leucocyte Antigens (HLA), could result in activation of the immune system. The absence of additional immune suppressive therapies, which in organ transplantation is a necessity to prevent graft rejection, could contribute to the rejection of valvular tissue. Consequently, rejection of valve allografts eventually causes tissue destruction and structural deterioration which ultimately results in functional failure of the valve grafts. Although previous studies have demonstrated the immunogenicity of valve allografts in vitro and in vivo [134, 143], the kinetics of cellular immune response in patients receiving a donor valve graft has not been studied thoroughly. Especially the involvement of Helper T (HTL) and Cytotoxic T lymphocytes may be of interest since these cells are associated with rejection of allografts [see 1.3.5]. In addition, the effect of donor-specific cellular reactivity on the valve function and structure remained unclear.

The general objective of the studies described in this thesis is to investigate the cellular immunological aspects of clinical and experimental valve allograft transplantation. Additionally, the consequence of such immune activation on the structural and functional properties of the valve allografts is investigated by using an experimental in vivo model. The clinical related studies are presented in part one of this thesis followed by descriptions of the experimental rat studies in part two of the thesis.

The first subject concerns the clinical results of cryopreserved human valve allografts implantation in the right ventricular outflow tract (RVOT) reconstruction. In this study a retrospective evaluation of a single center experience is reported. Statistical analysis regarding survival, allograft related re-operations and events are made including regression analysis for identification of risk factors. (**chapter 2**)

The second study describes the immunohistochemical analysis of both aortic and pulmonary human heart valve allografts regarding the presence of cells which are known to have immune stimulatory capacity. Additionally, the effect of cryopreservation on the structure and antigenicity of valve allografts is presented and discussed in **chapter 3**.

Because the process of transplant rejection is based on various regulator (helper T lymphocytes) and effector (cytotoxic T lymphocytes) mechanisms, measuring these cellular parameters in peripheral blood of valve allograft recipients could provide evidence for destructive immune activation which is associated with graft rejection. The kinetic and donor-specificity of interleukin-2 (IL-2) producing Helper T-lymphocyte frequencies in peripheral blood of valve allograft recipients was analyzed and presented in **chapter 4**. In a comparable study, described in **chapter 5**, the frequencies of cytotoxic T lymphocytes precursors (CTLp) were determined in the peripheral blood of valve allograft recipients taken at chronological intervals after graft implantation. Special attention was given to the difference in the avidity of these cells for donor HLA class I antigens. Additionally, **chapter 6** describes the difference in kinetics of circulating CTLp frequencies between adult and pediatric valve allograft recipients.

In order to evaluate the effect of donor-reactive immune activation in regard of valve allograft function and tissue morphology, a novel aortic valve transplantation model in the rat has been developed and evaluated (**chapter 7**). While previous heterotopic implantation models may include non-immunological factors which may hinder objective mediation of the structural and functional outcomes, the new implantation model may not have these pitfalls. Using this rat model, the functional and histo-pathological consequence of fresh allogeneic aortic valve transplantation is described in **chapter 8**. In **chapter 9** the anti-donor cellular reactivity is measured in rats receiving fresh aortic valve transplantation. The frequency of IL-2 producing Helper T lymphocytes in peripheral blood and spleen was determined in relation to valve dysfunction and cellular infiltration in the valve allografts. The effect of cryopreservation on the immunogeneicity and valve function was investigated using the rat model together with immunohistochemical analysis of monocytic infiltrates and the faith of donor cells in aortic valve allografts explanted early (7 or 21 days) after transplantation (**chapter 10**).

In **chapter 11** the observations from the studies are discussed in regard of the potential relevance in clinical valve allograft implantation. Finally, in **chapter 12**, a summary is given of the results and conclusions of the previous chapters followed by a Dutch version of the summary.

References

1. Lam CR, Aram HH, Mennell ER. An experimental study of aortic valve homografts. Surg Gynecol Obstet 1952;94:129-31
2. Murray G. Homologous aortic valve segment transplants as surgical treatment for aortic and mitral insufficiency. Angiology 1956;7:446-51
3. Kerwin AG, Lenkei SC, Wilson DR. Aortic valve homograft in the treatment of aortic insufficiency. Report of nine cases with one followed for 6 years. N Engl J Med 1962;266:852-4
4. Duran CG, Gunning AJ. A method for placing a total homologous aortic valve into the subcoronary position. Lancet 1962;2:488
5. Ross DN. Homograft replacement of the aortic valve. Lancet 1962;2:447
6. Barrat-Boyes BG. Homograft aortic valve replacement in aortic incompetence and stenosis. Thorax 1964;19:131-5
7. Ross DN, Somerville J. Correction of pulmonary atresia with a homograft aortic valve. Lancet 1966;2:1446-7
8. Yacoub MH, Kittle CF. A new technique for replacement of the mitral valve by a semilunar valve homograft. J Thoracic Cardiovasc Surg 1969;58:859-69
9. Lao GKH, Robles A, Cherian A, Ross DN. Surgical treatment of prosthetic endocarditis. J Thorac Cardiovasc Surg 1984; 87:712-5
10. Eguchi S, Asano KI. Homograft of pulmonary artery or ascending aorta with valve as right ventricular outflow. J Thorac Cardiovasc Surg 1968;56:413-5
11. Seki S, Rastelli GC, McGoon DC, Titus J. Replacement of the pulmonary artery with a pulmonary arterial homograft. J Thorac cardiovasc Surg 1970;60:853-8
12. Livi U, kay P, Ross D. The pulmonary homograft: an improved conduit for RVOT reconstruction. Circulation 1986;74(suppl 2):250
13. Campbell DN, Clarke DR. Use of the allograft aortic valved conduit. Ann Thorac Surg 1990;50:320-2
14. Robicsek F. Transplantation of heart valves. Orvosi Hetilap 1953;25:1-4
15. Pomar JL, Mestres CA. Tricuspid valve replacement using a mitral homograft: surgical technique and initial results. J Heart Valve Dis 1993;2:129-37
16. Acar C, Forge A, Ramsheyi A. Mitral valve replacement using a cryopreserved mitral homograft. Ann Thorac Surg 1994;57:746-8
17. Barratt-Boyes BG, Roche AHG, Brandt PW et al. Aortic homograft valve replacement. A long-term follow-up of an initial series of 101 patients. Circulation 1969;40:763-75
18. Malm JR, Bowman FO, Harris PD, Kovalik ATW. An evaluation of aortic homografts sterilized by electron-beam energy. J Thorac cardiovasc Surg 1967;54:471-5
19. Longmore DB, Lockey E, Ross DN, Pickering BN. The preparation of aortic valve homografts. Lancet 1966;2:463-4
20. Barrat-Boyes BG, Roch AHG, Subramanyan R et al. Long-term follow-up of patients with the antibiotic-sterilized aortic homograft valve inserted free-handed in the aortic position. Circulation 1987;75:768-77
21. O'Brien MF, Stafford EG, Gardner MAH et al. A comparison of aortic valve replacement with viable cryopreserved and fresh allograft valves, a note on chromosomal studies. J Thorac Cardiovasc Surg 1987;94:812-23
22. Angell WW, Angell JD, Oury JH et al. Long-term follow-up of viable frozen aortic homografts. J Thorac cardiasc Surg 1987;93:815-22

23. Parker R. An International survey of allograft banks. In: Cardiac valve allografts. Yankah AC, Yacoub MH, Hetzer R, Eds. Darmstadt: Steinkoff, New York: Springer Verlag, 1997
24. De Boer J, Hordijk W, Hogenbirk-Feller JPM et al. Protocol orgaan/weefseldonatie. Nederlandse Transplantatie Vereniging. Dec. 1997
25. Thijssen HJM, Bos E, Persijn GG. Een centrale hartkleppenbank voor transplantatie van humane hartkleppen. Ned Tijdschr Geneeskd 1991; 135:2116-20
26. Stegmann APA, Bogers AJJC. Annual Report , Heart Valve Bank Rotterdam 1999
27. Vongpatanasin W, Hillis LD, Lange RA. Prosthetic heart valves. N Engl J Med 1996;335:407-16
28. Jones EL, Weintraub WS, Craver JM et al. Ten-year experience with the porcine biothhetic valve: interrelationship of valve survival and patient survival in 1,050 valve replacements. Ann Thorac Surg 1990:49:370-83
29. Grunkemeier GL, Jamieson WR, Miller DC, Starr A. Actuarial versus actual risk of porcine structural valve deterioration. J Thorac Cardiovasc Surg 1994;108:709-18
30. Fann JI, Miller DC, Moore KA et al. Twenty-year clinical experience with porcine bioprotheses. Ann Thorac Surg 1996;62:1301-11
31. Cohn LH, Lipson W. Selection and complications of cardiac valvular prostheses. In: Baue AE, Geha AS, Hammond GL, Laks H, Naunheim KS, editors. Glenn's Thoracic and cardiovascular Surgery. Vol 2. 6th ed. Stamford (CT): Appleton & lange; 1996. Pp 2043-55
32. Edmunds LH, Clark RE, Cohn LH et al. Guidelines for reporting morbidity and mortality after cardiac valvular operations. J Thorac cardiovasc Surg 1996; 112:708-11
33. Mc Giffin DC. Invited letter concerning: Leaflet viability and the durability of the allograft aortic valve. J Thorac Cardiovasc Surg 1994;108:988-9
34. Kirklin JK, Smith D, Novick W et al. Long-term function of cryopreserved aortic homografts: a ten-year study. J Thorac Cardiovasc Surg 1993;106:154-65
35. O'Brien MF, Stafford EG, Gardner MA et al. Allograft aortic valve replacement: long-term follow-up. Ann Thorac Surg 1995;60(Suppl):S65-S70
36. Doty DB, Michielon G, Wang ND et al. Replacement of the aortic valve with cryopreserved aortic allograft. Ann Thorac Surg 1993; 56:228-35
37. Willem TP, Takkenberg JJ, Steyerberg EW et al. Human tissue valves in aortic position: Determinants of reoperation and valve regurgitation. Circulation 2001;103:1515-1521
38. Yacoub MH, Rasmi NR, Sundt TM et al. Fourten-year experience with homovital homografts for aortic valve replacement. J Thorac cardiovasc Surg 1995;110:186-95
39. Clarke DB, Campbell DN, Hayward AR et al. Degeneration of aortic valve allografts in young recipients. J Thorac Cardiovasc Surg 1993;105:934-42
40. Gallo R, Kumar N, Prabhakar G et al. Accelerated degeneration of aortic homograft in an infant. J Thorac Cardiovasc Surg 1994;107:1161-2
41. Hawkins JA, Bailley WW, Dillon T, Schwartz DC. Midterm results with cryopreserved allograft valved conduits from the right ventricle to the pulmonary arteries. J Thorac Cardiovasc Surg 1992;104:910-6
42. Niwaya K, Knott-Craig CJ, Lane MM et al. Cryopreserved homograft valves in the pulmonary position: Risk analysis for intermediate-term failure. J Thorsc Cardiovasc Surg 1999;117:141-7
43. Yankah AC, Alexi-Meskhishvili V, Weng Y et al. Performance of aortic and pulmonary homografts in the right ventricular outflow tract in children. J Heart Valve Dis 1995;4:392-5
44. Weipert J, Meisner H, Mendler N et al. Allograft implantation in pediatric cardiac surgery: Surgical experience from 1982 to 1994. Ann Thorac Surg 1995;60:S95-100

45. Perron J, Moran AM, Gauvreau KL. Valved homograft conduit repair of the right heart in early infancy. Ann Thorac Surg 1999;68:542-8
46. Clark DR, Bishop DA. Allograft degeneration in infant pulmonary valve allograft recipients. Eur J Cardio-thorac Surg 1993;7:365-70
47. Yankah AC, Alexi-Meskhishvili V, Weng Y et al. Accelerated degeneration of allografts in the first two years of life. Ann Thorac Surg 1995;60:S71-7
48. Baskett RJ, Ross DB, Nanton MA et al. Factors in the earily failure of cryopresereved homogaft pulmonary valves in children: preserved immunogeneicity? J Thorac Cardiovasc Surg 1996;112:1170-79
49. Introduction to the immune system. In Immunology. Roitt IM, Brostoff J, Male DK. Eds. Mosbey Barcelona Grafos SA. 1996
50. Clevers H, Alarcon B, Wileman T, Terhorst H. The T-cell receptor/CD3 complex: a dynamic protein ensemble. Ann Rev Immunol 1988;6:629
51. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-92
52. Sharrock CEM, Man S, Wanachiwanawin W, Batchlor JR. Analysis of the T cell repertoire in man. Transplantation 1987;43:699-705
53. Bernard A, BoumsellL, Dausset j, Milstein C, Schlossman SF. eds. Leukocyte differentiation antigens detected by monoclonal antibodies. Berlin: Springer-Verlag, 1984
54. Salter RD, Benjamin RJ, Wesley PK et al. A binding site for T cell co-receptor CD8 on the α3 domain of HLA-A2. Nature 1990;345:41
55. Koning R, Huang LH, Germain RN. MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. Nature 1992;356:799
56. Connolly JM, Hansen TH, Ingold AL, Potter TA. Recognition by CD8 on cytotoxic T lymphocytes is ablated by several substitutions in the class 1 α- domain: CD8 and the T-cell receptor recognize the same class I molecule. Proc Natl Acad Sci USA 1990;87:2137
57. Kupfer A, Singer SJ, Janeway CA Swain SL. Coclustering of CD4 (L3T4) molecule with the T cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. Proc Natl Acad Sci USA 1987;84:5888
58. de Vries JE, Yssel H, Spits H. Interplay between TCR/CD3 complex and CD4 or CD8 in the activation of cytotoxic T lymphocytes. Immunol 1985;109:119
59. Kim DT, Rothbard JB, Blomm DD, Fathman CG. Quantitative analysis of T cell activation. Role of TCR/ligand density and TCR affinity. J Immunol 1996;156:2737
60. Bachmann MF, Sebzda E, Kundig TM et al. T cell responses are governed by avidity and co-stimulatory thresholds. Eur J Immunol 1996;26:2017
61. Biddison WE, Shaw S. CD4 expression and functin in HLA class II specific T cells. Immunol Rev 1989;109:5
62. MacDonald HR, Glasebrook AL, Bron C, Kelso A, Cerottini JC. Clonal heterogeneity in the functional requirements for Lyt-2/3 molecules on cytolytic T lymphocytes (CTL) possibly implications for the affinity of CTL antigen receptors. Immunol Rev 1982;68:89
63. Bluestone JA. New perspectives of CD28-B7-mediated T cell co-stimulation. Immunity 1995;2:255
64. Breuning MH, van den Berg-Loonen PM, Bernini LF et al. Localizationof HLA on the short arm of chromosome 6. Human Genet 1977;37:131
65. Amos DB. Human Histocompatibility Locus HL-A. Science 1968;159:659-66
66. Bodmer JG, Marsh SGE, Albert ED et al. Nomenclature for factors of the HLA system 1996. Human Immunol 1997;53:98
67. Thorsby E. The role of HLA in T-cell activation. Hum Immunol 1984;9:1-11

68. Schwarz RH. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annual Rev Immunol 1985;3:237
69. Bjorkman PJ, saper MA, Samrauoi B et al. Structure of the human class I histocompatibility antigen, HLA-A2. Nature 1987;329:506
70. Bjorkman PJ, Saper MA, Samrauoi B et al. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature 1987;329:512
71. Brown JH, Jardetzky TS, Gorga JC et al. Three-dimensional structure of the human class II histocompatibility complex antigen HLA-DR1. Nature 1993;364:33
72. Stern LJ, Brown JH, Jardetzky TS et al. Crystal structure of human class II MHC protein HLA-DR1 complex with an influenza virus peptide. Nature 1994;368:215
73. Harding CV. Pathways of antigen processing. Curr Opin Immunol 1993;3:3-9
74. Koopmann JO, Hammerling GJ, Momburg F. Generation, intracellular transport and loading of peptides associated with MHC class I molecules. Curr Opin Immunol 1997;9:80-88
75. Pieters J. MHC class II restricted antigen presentation. Curr Opin Immunol 1997;9:89-96
76. Gould S, Auchincloss Jr. H. Direct and indirect recognition: The role of MHC antigens in graft rejection. Immunol Today 1999;20:77-82
77. Fisher-Lindahl K and wilson B. Histocompatibility antigen activated cytotoxic T lymphocytes. II Estimates of the frequency and specificity of precursors. J Exp Med 1977;145:508
78. Braun MY, McCormack A, Webb G and Batchelor JR. Mediation of acute but not chronic rejection of MHC-incompatible rat kidney grafts by alloreactive CD4 T cells activated by the direct pathway of sensitization. Transplantation 1993;55:177
79. Nijman HW, Kleijmeer MA, Ossevoort MA et al. Antigen capture and major histocompatibilty class II compartments of freshly isolated and cultured human blood dendritic cells. J Exp Med 1995;182:163
80. Rock KL, Rothstein L, Gamble S, Fleischacker C. Characterization of antigen0presenting cells that present exogenous antigens in association with class I MHC molecules. J Immunol 1992;150:438
81. Bevan MJ. Antigen presentation to cytotoxic T lymphocytes in vivo. J Exp Med 1995;182:639
82. Adams PW, Lee HS, Waldman WJ et al. Alloantigenicity of human endothelial cells. III. Quantitated indirect presentation of endothelial alloantigens to human helper T lymphocytes. Transplantation 1994;58:476
83. Kalhs P, White JS, Gervassi A, Storb R, Bean MA. In vitro recall of proliferative and cytolytic responses to minor histocompatibility antigens by dendritic cell enriched canine peripheral blood mononuclear cells. Transplantation 1995;59:112
84. Parker DC. T cell-dependent B cell activation. Annu Rev Immunol 1993;11:331
85. Croft M. Activation of native, memory and effector T cells. Curr Opin Imunol 1994;6:431
86. Lechler RI and Batchelor JR. Restoration of immunogeneicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. J Exp Med 1982;155:315
87. Kissmeyer-Nielsen F, Olsen S, Posberg-Petersen V, Fjeldborg O. Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. Lancet 1966;2:662
88. Billingham ME, Carry NR, Hammond ME et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart rejection study group. J Heart Transplant 1990;9:587
89. Häyry P, Isoiemi H, Yilmaz S et al. Chronic allograft rejection. Immunol Rev 1993;134:33
90. Medawar PB. The behavior and fate of skin autografts and skin homografts in rabbits. J Anat 1945;79:157

91. Porter KA, Joseph NH, Rendall JM et al. The role of lymphocytes in the rejection of canine renal homotransplants. Lab Invest 1964;13:1080
92. McKenzie IFC. Alloaggression. Transplant Proc 1983;15:269
93. Carter LL, Dutton RW. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. Curr Opin Immunol 1996;8:336
94. Swain SL. T cell subsets and the recognition of MHC class. Immunol rev 1983;74:129
95. Bach FH, Bach ML, Sondel PM. Differential function of major histocompatibility complex antigens in T cell activation. Nature 1976;259:273
96. Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; Inhibition by iso-antibody and drugs. Immunol 1968;14:181
97. Teh HS, Harley E, Phillips RA, Miller RG. Quantitative studies on the precursors of cytotoxic lymphocytes. J Immunol 1977;118:1049-55
98. Man S, Lechler RI, Batchelor JR, Sharrock C. Individual variation in the frequency of HLA class II-specific cytotoxic T lymphocyte precursors. Eur J Immunol 1990;2:847-53
99. Zhang L, Li S, Vandekerckhove BAE et al. Analysis of cytotoxic T cell precursor frequencies directed against individual HLA-A and HLA-B alloantigens. J Immunol Meth. 1989;121:39-46
100. Irschick EU, Hladik F, berger M et al. Clonal reduction of CTLp and acquired allograft tolerance in various human transplantation models. Transplant Proc 1990;22:1869
101. Steinmann J, Leimenstoll G, Engemann R et al. Clinical relevance of cytotoxic T-cell precursor (CTLp) frequencies in allograft recipients. Transplant Proc. 1990;22:1873-74
102. Dallman MJ, Wood KJ, Morris PJ. Specific cytotoxic T cells are found in the nonrejected kidneys of blood-transfused rats. J Exp Med 1987;165:566-72
103. Ouwehand AJ, Baan CC, Roelen DL, et al. The detection of cytotoxic T cells with high affinity receptors for donor antigens in the transplanted heart as prognostic factor for graft rejection. Transplantation 1993;56:1223-30
104. Van Emmerik NEM, Vaessen LMB, Balk AHMM et al. Progressive accumulation of CTL with high avidity for donor antigens during the development of acute cardiac rejection. Transplantation 1996;62:529-36
105. Van Emmerik NE, Vaessen LM, Knoop CJ et al. Kinetics of circulating cytotoxic T lymphocyte precursors that have a high avidity for donor antigens: correlation with the rejection status of the human cardiac allograft. Transpl Immunol 1998;6:153-60
106. Liu Y, Linsley PS. Costimulation of T-cell growth. Curr Opin Immunol 1992;4:265-69
107. Young NT, Roelen DL, Dallman MJ et al. Enumeration of human alloreactive helper T lymphocyte precursor frequencies by limiting diluyion analysis of interleukin-2 production. J Immunol Methods 1996;195:33-41
108. Schwarer AP, Jiang YZ, brookes PA, et al. Frequency of anti-recipient alloreactive helper T-cell precursors in donor blood and graft-versus-host disease after HLA-identical sibling bone-marrow transplantation. Lancet 1993;341:203-14
109. Debruyne LA, Ensley RD, Olsen SL, et al. Increased frequency of alloantigen-reactive helper T-lymphocytes is associated with human cardiac allograft rejection. Transplantation 1993;56:722-29
110. Veassen LMB, Daane CR, Maat APWM et al. T helper frequencies in peripheral blood reflect donor-directed reactivity in the graft after clinical heart transplantation. Clin Exp Immunol 1999;118:473-79
111. Mohri H, Reichenbach DD, Barnes RW et al. Studies of the antigenicity of the homologous aortic valve. J Thorac Cardiovasc Surg 1967;54:564-72

112.Baue AE, Donowick WJ, Blakemore WS. The immunologic response to heterotopic allovital aortic valve transplants in presensitized and nonsensitized recipients. J Thorac cardiovasc Surg 1968;56:775-89

113.Buch WS, Kosek JC, Angell WW. The role of rejection and mechanical trauma on valve graft viability. J Thorac cardiovasc Surg 1971;62:696-706

114.Heslop BF, Wilson SE, Hardy BE. Antigenicity of aortic valve allografts. Ann Surg 1973;177:301-6

115.Gonzalez-Lavin L, Bianchi J, Graf D et al. Degenerative changes in resh aortic root homografts in a canine model: evidence of an immunologic influence. Transplantation Proc 1988;1:815-19

116.Tiede A, Timm C, Bernhard A et al. Studies on the antigenicity of vital allogeneic valve leaflet transplants in immunologically controlled strain combinations. Transplantation 1978;26:391-95

117.El Khatib H, lupinetti FM. Antigenicity of fresh and cryopreserved rat valve allografts. Transplantation 1990; 49:765-67

118.Lupinetti FM, Cobb S, Kioschos HC et al. Effect of immunological differences on rat aortic valve allograft calcification. J Card Surg 1992;7:65-70

119.Lupinetti FM, Tsai TT, Kneebone JM et al. Effect of cryopreservation on the presence of endothelial cells on human valve allografts. J Thorac cardiovasc Surg 1993;106:912-7

120.Yankah AC, Wottge HU, Muller-Ruchholtz W. Prognostic importance of viability and a study of a second set allograft valve: an experimental study. J Card Surg 1988;3:263-70

121.Moustapha A, Ross DB, Bittira B et al. Aortic valve grafts in rat : evidence for rejection. J Thorac cardiovasc Surg 1997;114:891-902

122.Green MK, Walsh MD, Dare A et al. Histologic and immunohistochemical responses after aortic valve allografts in the rat. Ann Thorac Surg 1998;66:S216-20

123.Legare JF, Lee TD, Creaser K, Ross DB. T lymphocytes mediate leaflet destruction and allograft aortic valve failure in rats. Ann Thorac Surg 2000;70:1238-45

124.Zhuo ZY, Golshani SD, Wellisz T et al. A new model for heterotopic aortic valve transplantation. Transplantation 1997;64:228-32

125.Zhao XM, Green MK, Frazer IH et al. Donor-specific immune response after aortic valve allografting in the rat. Ann Thorac Surg 1994;57:1158-63

126.Lupinetti FM, Christy JP, King DM et al. Immunogenicity, antigeneicity and endothelial viability of aortic valves preserved at 4oC in a nutrient medium. J Cardiol Surg 1991;6:454-61

127.Muller-Hermelink HK, Yankah AC. Immunohistopathology of cardiac valve allograft explants. In: Yankah AC, Hetxer R, Miller DC, Ross DN, Sommerville J, Yacoub MH, editors. Cardiac valve allografts 1962-87. New York: Springer-Verlag, 1988:89-94

128.Shaddy RE, Tani LY, Sturtevant JE et al. Effects of homograft blood type and anatomic type on stenosis, regurgitation and calcium in homografts in the pulmonary position. Am J Cardiol 1992;60:392-3

129.Balch CM, Karp RB. Blood group compatibility and aortic valve allotransplantation in man. J Thorac cardiovasc Surg 1975;70:256-59

130.Yacoub MH. Applications and limitations of histocompatibility in clinical cardiac valve allograft surgery. In Yankah AC, Hetxer R, Miller DC, Ross DN, Sommerville J, Yacoub MH, editors. Cardiac valve allografts 1962-87. New York: Springer-Verlag, 1988:95-102

131.Simon A, Wilhelmi M, Steinhoff G et al. Cardiac valve endothelial cells: relevance in the long-term function of biologic valve prostheses. J Thorac cardiovasc Surg 1998;116:609-16

132.Salomon RN, Friedman GB, Callow AD et al. Cryopreserved aortic homografts contain viable smooth muscle cells capable of expressing transplantation antigens. J Thorac Cardiovasc Surg 1993;106:1173-80

133.Page CS, Holloway N, Smith H, Yacoub M, Rose ML. Alloproliferative response of purified CD4+ and CD8+ T cels to endothelial cells in the absence of contaminating accessory cells. Transplantation 1994;57:1628

134.Hoekstra FM, Knoop CJ, Aghai Z et al. Stimulation of immune-competent cells in vitro by human cardiac valve-derived endothelial cells. Ann Thorac Surg 1995;60:S131-4

135.Yacoub M, Suitters A, Khaghani A et al. Localization of major histocompatibility complex (HLA, ABC and DR) antigens in aortic homografts. In: Bodnar E, Yacoub M (eds): Biologic and bioprosthetic valves. Proceedings of the third interbnational symposium. New York, York medical Books 1986:65-72

136.Bobryshev YV, Lord RSA. S-100 positive cells in human arterial intima and in atherosclerotic leasions. Cardiovasc Res 1995;29:689-96

137.Nakajima T, watanabe S, Sato Y et al. An immunoperoxidase study of S-100 protein distribution in normal and neoplastic tissue. Am J Surg Pathol 1982;6:715-27

138.Toriyama K, Wen DR, Paul E, Cochran AJ. Variations in the distribution, frequency and phenotype of langerhans cells during the evolution of malignant melanoma of the skin. J Invest Dermatol 1993;100:269-73S

139.Strutton G, Hogan P, Green M. surface antigens on dendritic cells in human aortic valves. Abstract 8th International symposium on cardiac bioprostheses 2000. p.43

140.Hoekstra FM, Knoop CJ, Jutte NH et al. Effect of cryopreservation and HLA-DR matching on the cellular immunogeneicity of human cardiac valve allografts. J Heart Lung Transplant 1993;13:1095-8

141.Hoekstra FM, Witvliet M, Knoop CJ et al. Immunogenic human leucocyte antigen class II antigens on human cardiac valves induce specific alloantibodies. Ann Thorac Surg 1998;66:2022-6

142.Smith JD, Ogino H, Hunt D et al. Humoral immune response to human aortic valve homografts. Ann Thorac surg;60:s127-30

143.Hogan P, Duplock L, Green M et al. Human aortic valve allografts elicit a donor-specific immune response. J Thorac Cardiovasc Surg 1996;112:1260-67

144.Hawkins JA, Breinholt JP, Lambert LM et al. Class I and class II anti-HLA antibodies after implantation of cryopreserved allograft material in pediatric patients. J Thorac Cardiovasc Surg 2000;119:324-30)

145.Schutz A, Fischlein T, Breuer M et al. Cytoimmunological monitoring after homograft valve replacement. Eur J cardio-thorac Surg 1994;8:609-12

146.Jutte NH, Hop WC, Daane R et al. Cytoimmunological monitoring of heart transplant recipients. Clin Transplantation 1990;4:297-300

147.Lupinetti FM, King DM, El-Khatib HE et al. Immunogeneicity of aortic valve allografts does not correlate with the precence of antige. J Am Coll Cardiol 1991;17:213A.

148.Mitchell RN, Jonas RA, Schoen FJ. Structure-function correlations in cryopreserved allograft cardiac valves. Ann Thorac Surg 1995;60:S108-13

149.Goffin YA, Henriques de Gouveia R, Szombathelyi T et al. Morphologic study of homograft valves before and after cryopreservation and after short-term implantation in patients. Cardiovasc pathol 1997;6:35-42

150.Koolbergen DR, hazekamp MG, Kurvers M et al. Tissue chimerism in human cryopreserved homograft valve explants demonstrated by in situ hybridization. Ann Thorac Surg 1998;66:S225-32

151.Vogt PR, Stallmach T, Niederhauser U et al. Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infants and adults. Eur J cardio-thorac Surg 1999;15:639-45

152.Hoekstra FM, Knoop CJ, Vaessen LM et al Donor-specific cellular immune response against human cardiac valve allografts. J Thorac cardiovasc Surg 1996;112:281-6

PART I

PATIENT RELATED STUDIES

CHAPTER 2

Right ventricular outflow tract reconstruction with an allograft conduit

C.G Gerestein, J.J.M. Takkenberg, F.B.S. Oei, A.H. Cromme-Dijkhuis, S.E.C. Spitaels, L.A. van Herwerden, E. W. Steyerberg and A.J.J.C. Bogers.

Published in Annals of Thoracic Surgery, 2001;71 911-8

Abstract

Allograft conduits are used for reconstruction of the right ventricular outflow tract (RVOT) in patients with congenital heart disease and in the pulmonary autograft procedure. A retrospective evaluation of our experience with the use of allograft conduits for reconstruction of the right ventricular outflow tract was conducted.

Between August 1986 and March 1999, 316 allografts (246 pulmonary, 70 aortic) were implanted in 297 patients for reconstruction of the right ventricular outflow tract. Main diagnostic groups were aortic valve pathology (N=112, 35%), tetralogy of Fallot (N=71, 22 %) and pulmonary atresia with ventricular septal defect (N=46, 14%). Kaplan-Meier analyses were done for survival, valve-related reoperation and valve-related events. In addition, Cox regression analysis was used for evaluation of potential risk factors.

Mean age at operation was 18 years (range: 7 days to 61 years). Mean follow-up was 4 years (range: 2 days to 12 years). Twelve patients (4%) died within 30 days after operation. Patient survival was 90% (95% confidence interval (CI) 86-94%) at 5 years and 88% (95% CI 83 to 94%) at 8 years. Twenty-four reoperations were required for allograft dysfunction in 23 patients; 21 allografts were replaced. Freedom from valve-related reoperation was 91 % (95% CI 86-95) at 5 years and 87% (95% CI 81 to 93%) at 8 years. Twenty-nine valve-related events were reported (2 deaths, 24 reoperations, 2 balloon dilatations, and 1 endocarditis). Freedom from valve-related events was 90% (95%CI 85-94%) at 5 years after implantation, and 84% (95% CI 77 to 91%) at 8 years. Risk factors for accelerated allograft failure were extra-anatomic position of the allograft ($p=0.03$; hazard ratio 9.7) and the use of an aortic allograft ($p=0.02$; hazard ratio, 2.4).

Right ventricular outflow tract reconstruction with an allograft conduit has good medium- term results, although progression of allograft degeneration is noted. Aortic allografts should preferably not be used for reconstruction of the RVOT.

Introduction

Reconstruction of the right ventricular outflow tract (RVOT) is performed in patients with congenital heart disease when there is no adequate continuity between right ventricle and pulmonary circulation. In 1966 Ross and Somerville introduced the aortic allograft in RVOT reconstruction [1]. Results of pulmonary allograft implantation in the RVOT became available in the late 1980's [2]. Development of (cryo) preservation techniques has improved the availability and durability of allografts considerably. This has resulted in an ever-increasing application of allografts. For instance, allografts are also often used to reconstruct the RVOT during the pulmonary autograft procedure [3]. Nevertheless, a tendency for degeneration over the years is still apparent [4]. The latter may cause dysfunction leading to an overload on the right ventricle and may eventually necessitate reoperation.

Long-term results of RVOT reconstruction with allografts have been scarcely reported thus far. Stark and colleagues described 84%, 58%, and 31% freedom from conduit replacement at 5, 10, and 15 years, respectively [5]. Niwaya and associates reported 90% freedom from allograft failure after 5 years and 82% after 8 years [6]. In other series extra-anatomic position of the allograft and the use of aortic allografts were noted as risk factors for accelerated allograft failure [3,6-9]. We describe our experience with allograft conduits for reconstruction of the RVOT to contribute to improved knowledge on outcome after allograft implantation.

Materials and methods

Between August 1986 and March 1999, 316 allografts (246 pulmonary, 70 aortic) were implanted in 297 patients for reconstruction of the RVOT or to replace the pulmonary valve in a pulmonary autograft procedure at the University Hospital Rotterdam.

Table I. Primary diagnosis at time of allograft implantation (N=316).

Diagnosis	No. of Operations (%)
Aortic valve pathology	112 (35%)
Tetralogy of Fallot	71 (22%)
PA or PS, VSD	46 (15%)
Discordant ventriculo-arterial connection with PA or PS	39 (12%)
Common arterial trunk	34 (11%)
PA or PS with intact septum	11 (4%)
Aortic atresia with biventricular heart	3 (1%)
Total	316 (100%)

(PA = pulmonary atresia, PS= pulmonary stenosis, VSD= ventricular septal defect)

We excluded from this analysis an additional 13 right-sided allografts used in 11 patients with univentricular hearts.

Patient characteristics. Our series represents a heterogeneous group in which the common denominator was the need for a right-sided allograft conduit. Patients were classified according to their primary diagnosis (Table I). Main diagnostic groups were aortic valve disease (35%), tetralogy of Fallot (22%), and pulmonary atresia with ventricular septal defect (VSD) (15%). A first allograft was implanted in 296 patients, a second in 17, a third in 2, and a fourth in 1. Prior cardiac operation was performed in 200 patients (63%) of which 95 had previous RVOT reconstruction; their actual pathologic disorder is shown in Table II.

Table II. Actual pathology at time of allograft implantation after previous RVOT reconstruction related to primary diagnosis (N=95).

Actual pathology	All	Primary Diagnosis PA,PS VSD	PA or PS and intact septum	ToF	Common arterial trunk	Discordant venrticuloarterial connection with PA or PS
Transannulair patch dysfunction	43	1	4	36	1	1
Allograft dysfunction	19	4	1	6	5	3
Xenograft conduit dysfunction	16	3	-	3	4	6
Dysfunction after pulmonary valvotomy	9	-	4	5	-	-
Prostehtic non-valved conduit dysfunction	5	1	-	-	1	3
Allograft monocusp dysfunction	3	-	-	3	-	-
Total	**95**	**9**	**9**	**53**	**11**	**13**

PA= pulmonary atresia, PS=pulmonary stenosis, VSD= ventricular septal defect, ToF= Tetralogy of Fallot

Operative techniques. The surgical procedures were performed using standard cardiopulmonary bypass with moderate hypothermia, myocardial protection with crystalloid cardioplegia (St. Thomas solution), and in most cases topical cooling.

If associated intra-cardiac procedures were not required, the heart was kept beating and the reconstruction was done without cross-clamping of the aorta. Preparation of the allograft occurred according to protocol [10]. Using the interposition technique the allograft was sewn between the right ventricle and pulmonary artery in most cases (N= 311). In 5 patients the allograft was implanted between the right-sided left ventricle and the pulmonary artery. Distal anastomosis was made with a running polypropylene suture. Nineteen patients needed a distal extension to ensure proper connection. For this purpose an allograft patch (N=10), an autologous pericardial patch (N=3) or a prosthetic patch (N=6) was used. The proximal anastomosis was also made with a running polypropylene suture. A proximal extension of the allograft was necessary in 92 patients. In these cases an allograft patch (N=38), the anterior mitral valve leaflet of the aortic allograft (N=24), a pericardial patch (N=20) or a prosthetic patch (N=10) was used. In all cases attempts were made to implant the allograft away from the sternum to prevent compression or distortion.

Allograft properties. The Rotterdam Heart Valve Bank provided most of the allografts (N= 242) which were allocated by Bio Implant Services, Leiden, The Netherlands. Preparation and storage methods have been described earlier [10]. The National Heart Hospital, London, England, provided 16 fresh and 4 cryopreserved allograft conduits. The remaining allografts were shipped from the Hospital Clinic I, Barcelona , Spain (N=28), the Karolinska Homograft bank, Stockholm, Sweden (N=6), the Deutsches Herzzentrum, Berlin (N=19) and Herzzentrum Nord Rhein Westphalen, Bad

Oeynhausen (N=1), both Germany. Patient's body surface area was used as a guideline to determine the allograft diameter. No attempt was made to achieve ABO blood type or HLA type matching.

Follow-up. After implantation of the allograft, patients were seen at regular intervals by their cardiologists, with the exception of 17 patients who migrated to other countries or were living abroad. All follow-up data were collected retrospectively from hospital records. Follow-up was 95% complete. Allograft functional status was evaluated by physical examination, electrocardiography, echocardiography and if necessary cardiac catheterization. The day of implantation was considered as starting point of patient survival. End points in patient survival were death or last follow-up date. Patients lost to follow-up were censored at last date of follow-up (N=17; mean follow-up 3.2 years (SD 3.0, range 1 week-8 years). Starting point of allograft survival also was the day of implantation. End points were allograft dysfunction as defined by Edmunds and co-workers [11] or last follow-up date.

Data Analysis. Patient data were entered into a computerized relational database (Microsoft Access 97). All statistical analyses were done using SPSS 8.0 for Windows (Chicago, Ill, USA). Cumulative survival estimates were made at 5 and 8 years using the Kaplan-Meier method [12]. The log-rank test was used for univariate assessment of the effect of potential risk factors on patient survival, freedom from valve-related reoperation, and freedom from valve related events. To investigate independent risk factors for mortality and morbidity caused by allograft failure the Cox proportional hazard model was used. Risk factors were selected with a backward stepwise method (criteria for elimination: $p>0.20$). Young age at implantation ($<$ 4 years of age versus $\geq$ 4 years of age) and small allograft diameter ($<$ 23 mm versus $\geq$ 23 mm) were defined according to their distribution in the patient population (Fig 1A, Fig 1B).

After evaluation of the frequency distribution of donor age, young donor age was arbitrarily set at less than 30 years of age (Fig 1C). with regard to implantation position all autograft procedures were labeled as anatomic, and any other allograft implantation for reconstruction of the RVOT was labeled as extra-anatomic. Young age at time of implantation ($<$4 years), small allograft diameter ($<$23 mm), extra-anatomical position of the allograft, ABO incompatibility between donor and recipient, young donor age ($<$30 years), and an aortic allograft were considered to be potential risk factors for allograft dysfunction [4,6-9). Association between potential risk factors was studied calculating Pearson's correlation coefficient (two-tailed testing).
To further investigate the effect of young patient age, subanalyses were performed of patients younger than 4 years and patients younger than 1 year at the time of operation. Cumulative survival estimates were made for patient survival, valve-related reoperation and valve-related events.

Figure 1. frequency distribution of patient age at operation (A), Allograft diameter (B), and donor age (C)

Figure 1A

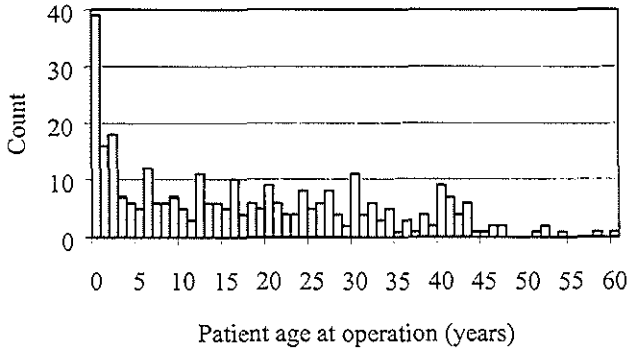


Figure 1B

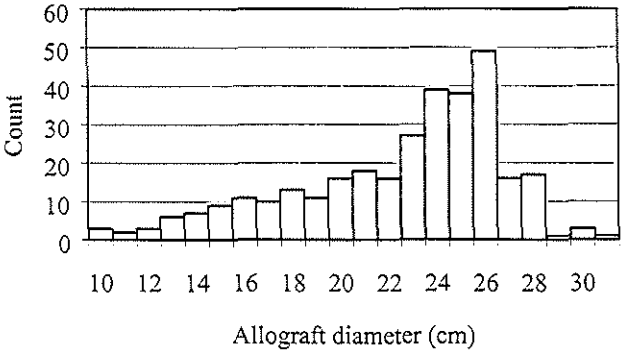
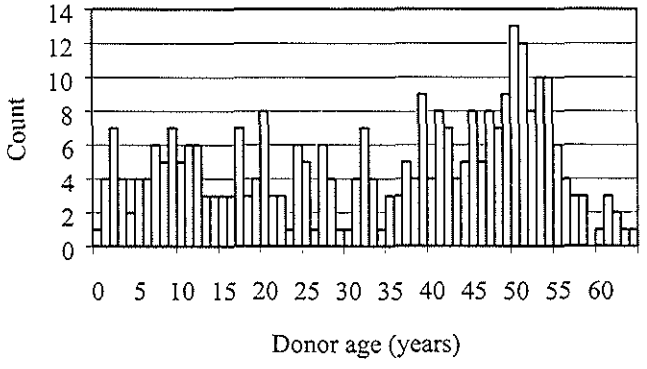


Figure 1C



Results

Patient characteristics. Mean (± SD) age at time of operation was 18 ± 15 years (median 16; range, 7 days to 61 years). Thirty-nine patients (12%) were aged less than 1 year, 78 patients (25%) less than 4 years. The patient group consisted of 180 men and 136 women (male-female ratio, 3:1) with a mean weight at operation of 42 ± 27 kg (median, 48; range: 2 to 111 kg) and a mean length of 1.37 ± 0.44 m (median, 158; range, 0.45 to 2.00 m).

Donor characteristics. The donor group consisted of 191 male and 117 female donors (male-female ratio 6:1) with a mean age of 33 ± 18 years (median, 38; range, 0 to 64 years). The characteristics of eight donors could not be traced. Mean allograft diameter was 22 ± 5 mm (median, 24; range, 10 to 31 mm). Of the 316 allografts, 300 were cryopreserved and 16 were fresh.

Follow-up. Follow-up ranged from 2 days to 12 years with a mean follow-up time of 4 years ± 3 (median, 3 years). Total number of patient years was 1209.

Survival. Twelve patients (4%) died within thirty days of operation. Causes of early death were heart failure (N=4), bleeding (N=4), hypoxic encephalopathy (N=1), respiratory insufficiency (N=1), pulmonary thromboembolism (N=1) and arrhythmia (N=1). All deaths were non-valve-related No allografts showed signs of degeneration at pathologic examination. Fifteen patients died later than 30 days after implantation. Two of these deaths were valve-related. In 1 patient calcification of the allograft valved conduit caused stenosis resulting in acute right heart failure. Endocarditis destroyed the allograft in another patient resulting in right ventricular failure. Causes of non-valve related late death were heart failure (N=6), respiratory insufficiency (N=2), sepsis (N=2), myocardial infarction (N=1), arrhythmia (N=1) and hypoxic encephalopathy (N=1). In the 13 non-valve-related late deaths, severe pulmonary regurgitation because of structural valvefailure was present at the last echocardiographic examination before death in 3 patients (2 died of right heart failure, 1 of arrhythmia) and moderate in 1 and mild pulmonary regurgitation was also present in 1 other patient. Furthermore, 1 patient with mild pulmonary stenosis and mild pulmonary regurgitation was noted. In the other 7 patients no pulmonary regurgitation or stenosis was present. Patient survival at one month was 96% (95% CI 94 to 98%) and 93 % (95% CI 90 to 96 %) at 1 year. Five and 8 year survival were respectively 90 % (95% CI 86 to 94 %) and 88 % (95% CI 83 to 94%) (Fig 2). Univariate analysis revealed that younger donor age was the only risk factor for death ($p=0.04$, hazard ratio 2.3, (95% CI 1.0 to 5.0)).

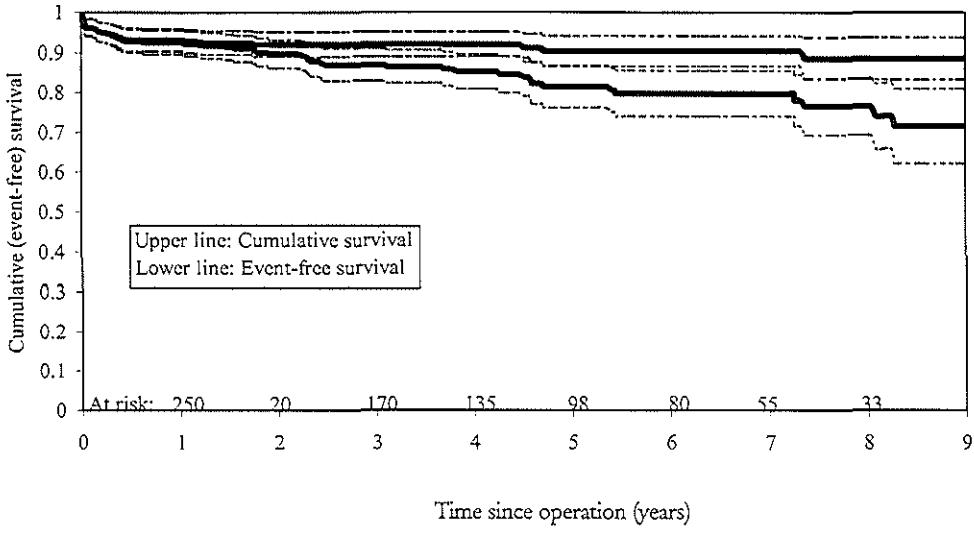


Figure 2. Actuarial cumulative survival and event-free survival (dotted line represent the 95% confidence interval

Valve-related events. During the follow-up 29 valve-related events were reported: 24 reoperations, 2 deaths, 2 balloon dilatations, and 1 case of endocarditis.

Twenty-four reoperations were performed for allograft dysfunction in 23 patients. Allograft dysfunction was related to structural valve failure in 21 patients, characterized by stenosis in 19 patients and regurgitation in the remaining 2. Eighteen of the explanted allografts showed calcification. One allograft, replaced for structural valve failure, was not calcified. A false aneurysm in one sinus was responsible for the regurgitation in this patient. Conduit failure was nonstructural in 5 patients. On two occasions a prosthetic extension of the conduit caused stenosis near the proximal anastomosis of the allograft. A pericardial patch and a surgical membrane obstructed the allograft in 2 other cases, and 1 patient suffered from supravalvular stenosis near the distal anastomosis. In 21 operations the allograft was replaced, in 2 patients extension material causing allograft stenosis was removed and, in 1 patient a pulmonary allograft patch was used for enlargement of the RVOT. Freedom from reoperation was 91 % (95% CI 86 to 95%) after 5 years and 87% (95% CI 81 to 93%) after 8 years. Log-rank testing revealed younger age at operation (<4 years) (p=0.02, HR 2.6, (95% CI 1.1 to 5.7)), smaller allograft diameter (<23 mm) (p=0.01, HR 3.5, (95% CI 1.4 to 9.1)), extra-anatomic position of the allograft (p=0.02, HR 10.1, (95% CI 1.4 to 74.7)), young donor age (< 30 years) (p=0.01, HR 3.5, (95% CI 1.3 to 9.5)) and, aortic allograft (p=0.008, HR 4.2, (95% CI 1.8 to 9.6)) as potential risk factors for valve-related reoperation. After multivariate testing extra-anatomic position (p=0.06, HR 7.2, (95% CI 0.94 to 54.9)) and the use of

an aortic allograft (p=0.006, HR 3.3, (95% CI 1.4 to 7.8)) turned out to be the most important risk factors for reoperation.

Besides the 24 valve-related reoperations, 5 more valve-related events were noted. The 2 valve-related deaths are described above. Two patients required balloon dilatation for allograft stenosis. The remaining valve-related event occurred in a patient who suffered from endocarditis 5 months after successful reoperation for a stenotic left pulmonary artery and recurrent ventrical septal defect. This endocarditis was successfully treated medically. Valve thrombosis, embolism and bleeding events were not observed during follow-up.

Ninety percent (95% CI 85 to 94%) of the patients were free from any valve-related event 5 years after implantation. After 8 years this percentage was reduced to 84 % (95 % CI 77 to 91%). Event-free survival 81% (95% CI 76 to 87%) at 5 years and 76% (95% CI 69 to 83%) at 8 years after operation) is displayed in Figure 2. Univariate analysis showed that young age at operation (<4 years) (p=0.02, HR 2.4, (95% CI 1.2 to 5.1)), younger donor age (<30 years) (p=0.01,HR 3.3, (95% CI 1.3 to 8.4)), extra-anatomic position of the allograft (p=0.01, HR 11.8, (95% CI 1.6-87.4)), small diameter (<23 mm) (p=0.004, HR 3.6, (95% CI 1.5 to 8.5)) and, an aortic allograft (p=0.005, HR 2.9, (95% CI 1.4 to 6.1)) were potential risk factors for the occurrence of valve-related events after implantation of the allograft. All potential risk factors for the occurrence of valve-related events were positively correlated (Pearson's R≥0.32; p<001). In particular patient age at operation was highly correlated with type of allograft used, donor age and allograft diameter (Pearson's R≥0.50; p<0.01). Extra-anatomic position (p=0.03, HR 9.7, (95% CI 1.3 to 72.6)) and aortic allograft (p=0.02, HR 2.4, (95 % CI 1.1 to 5.1)) were the most important independent risk factors found (Table III).

Sub-analysis young patient age at operation. To further investigate the importance of age at operation a subanalysis was performed for patients younger than 4 years at time of operation. This cutoff point was chosen after evaluation of the distribution of age in our patient population (Fig 1A). 78 patients were younger than 4 years at the time of operation. Main diagnostic groups were common arterial trunk (N=21, 27%) and pulmonary atresia with ventricular septal defect (N=22, 28%). Mean allograft diameter was 16 ± 3.1 mm (range, 10-23 mm) and mean donor age was 14 ± 13 years (range, 0-54 years).

The early mortality was 2 (2.6%) and late mortality concerned another 10 patients. Eleven reoperations had to be performed. Thirteen valve-related events were noted (1 death, 12 reoperations). Eight-year survival in this group was 84 % (95% CI 75 to 92%). Freedom from reoperation at 8 years was 75% (95% CI 60 to 90%). Freedom from valve-related events was 73% (95%CI 58 to 88%) at 8 years.

Table III. Freedom from valve-related events at 8 years stratified for risk factors (N=316).

Analyzed factor	Freedom from valve-related events	95 % CI	P value, UV	OR, UV (95% CI)	P value MV	OR, MV (95% CI)
Age at implantation:						
< 4years (N=78)	73%	56-88%	0.02	2.4	NS	
> 4 years (N=238)	88%	80-96%		(1.2-5.1)		
Allograft position:						
Extra-anatomical	80%	71-89%	0.01	11.8	0.03	9.7
(N=204)	94%	82-100%		(1.6-87.4)		(1.3-72.6)
Anatomical (N=112)						
Type of allograft:						
Aortic (N=70)	72%	60-86%	0.005	2.9	0.02	2.4 (1.1-5.1)
Pulmonary (N=246)	89%	80-98%		(1.4-6.1)		
Donorage:						
< 30 years (N=125)	76%	66-87%	0.01	3.3	NS	
> 30 years (N=183)	89%	77-100%		(1.3-8.4)		
Allograft diameter						
<23 mm (N=125)	75%	64-86%	0.004	3.6	NS	
>23 mm (N=191)	91%	80-100%		(1.5-8.5)		

(CI=Confidence interval, UV= univariate, MV= multivariate).

Univariate analysis did not reveal any risk factors for death, but the use of an aortic allograft tended to be associated with valve-related reoperation (p=0.08, HR 6.5, (95% CI 0.8 to 50.8)) and valve-related events (p=0.09, HR 3.7, (95% CI 0.8 to 16.6)). In addition, patients aged younger than 1 year at the time of operation were studied (N=39).

The main diagnostic groups were common arterial trunk (N=21, 54%) and pulmonary atresia with ventricular septal defect (N=4, 10%). Mean allograft diameter was 15 ± 2.4 mm (range, 10 to19 mm) and mean donor age was 8 ± 7 years (range, 0 to 33 years). The early mortality was 2 (5.1%) and late mortality concerned another 6 patients. Eight reoperations had to be performed. Nine valve-related events were noted (1 death, 8 reoperations). Eight-year survival in this group was 75 % (95% CI 61 to 89%). Freedom from reoperation at 8 years was 67% (95% CI 46 to 88%). Freedom from valve-related events was 65% (95%CI 44 to 86%) at 8 years. Univariate analysis did not reveal any risk factors for death, valve-related reoperation, or valve-related events.

Discussion

Allograft implantation is accepted as the most frequently used method to reconstruct the RVOT with a valved conduit [3,4,6,13]. However, the reported freedom from reoperation for conduit dysfunction ranges from 45 to 84% at 5 years [3,5,13]. In addition, series with good medium-term results report less satisfying results for the longer term, for example Stark and colleagues described 58% and 31% freedom from conduit replacement at 10 and 15 years, respectively [5]. The relatively young patient population, a large amount of aortic allografts used in the latter series, and use of noncryopreserved allografts in the early implantation period, may explain these results. In this regard, our patient survival of 90% at 5 years and 88% at 8 years is comparable with other studies [4,6,9,13]. Only 2 deaths were related to allograft dysfunction. We found a freedom from reoperation of 90% at 5 years and 86% at 8 years. Freedom from valve- related events was 90% and 84% at 5 and 8 years respectively. This is consistent with other reports, e.g. Niwaya and coworkers reported 90% freedom from allograft failure after 5 years and 82% after 8 years [6]. In an earlier report from our center, Willems and associates found 78% freedom from valve-related events in 5 years after operation [3]. It therefore appears that our results have improved somewhat in recent years. Ample experience with these operations in our center, and a relative increase of pulmonary autograft patients in our series are possible explanations for this improvement. Analysis of survival, freedom from reoperation and freedom from valve-related events in our patient group supports the adequacy of allografts for RVOT reconstruction. Despite the improvement of clinical results in patients undergoing more recent operations, we note progressive allograft dysfunction with increasing follow up of the patient population. Further long-term surveillance is therefore necessary.

Risk factors for allograft failure. Known independent risk factors for allograft failure are the use of aortic allografts and extra-anatomic position of the allograft [4,6-9]. These factors were confirmed by multivariate analysis in our study. In the RVOT, the pulmonary allograft is preferred above the aortic allograft, because apparently the aortic allograft in pulmonary position is more prone to degeneration. Albert and associates found a freedom from valve replacement or valve-related death in patients with an aortic allograft of 76% after 5 years compared with a 94 % freedom from valve replacement or valve-related death in patients with a pulmonary allograft [7]. Bando and colleagues reported 70% freedom from allograft failure in aortic allografts to 94% in pulmonary allografts after 5 years [8]. A lower content of elastic tissue and a lower amount of total calcium in the wall of the pulmonary allograft in comparison to the aortic allograft are argued to play a role in this difference [14].

Multivariate analysis revealed that allografts implanted in extra-anatomic position were more likely to fail than an allograft used in a pulmonary autograft procedure, where the allograft is implanted in

anatomic position in an anatomically normal heart. Extra-anatomic position has been recognized as a risk factor for allograft dysfunction by other authors as well [4,6,9].

Age at operation is said to be another important determinant of allograft survival [3,6,9,13]. This is however, not supported by the multivariate risk factor analysis in our present study. Allografts implanted at younger age tend to be more prone to degeneration than those implanted in older patients. More complex disease process and usage of allografts with small diameter from younger donors in this group are possible explanations for the difference in allograft failure between patient operated at younger (<4 years) and older ages. The strong correlation that was found between patient age at operation and the other potential risk factors in the multivariate model supports this hypothesis. The sub-analysis of young patient age at operation also illustrates the relationship between the following risk factors: young patient age at operation, young donor age and small allograft diameter. Patients operated on at a young age in general receive an allograft with a small diameter. The heart will outgrow the allograft after a few years, resulting in the need for reoperation. To prevent this some authors advise using an allograft with a relatively larger diameter [15]. However, implanting too large an allograft entails a risk for compression or kinking of the allograft.

A final explanation for limited allograft survival, especially in younger patients, may be accelerated immune-mediated deterioration of the allograft. Recently both Vogt and co-workers and Rajani and associates found histological evidence for immune-related structural damage of pulmonary allografts [16,17]. In as much as allograft implantation is performed without blood type ABO-antigens or Human Leukocyte Antigens (HLA) matching, a specific immunologic response of the valve recipient directed against donor antigens could be expected.

In an in vitro study, Hoekstra and colleagues have demonstrated that HLA class II (DR) discrepancy of human valve tissue could activate immune-competent cells. The same group found an increase of antidonor HLA antibodies and destructive cytotoxic T lymphocytes in the peripheral blood of allograft recipients [18]. Additionally, animal studies have clearly outlined the ability of this specific immune response to damage the freshly implanted allograft [19]. However, the clinical relevance of such donor-specific immune activation still remains uncertain. In the present study, blood type ABO-antigen mismatch was not a significant risk factor for allograft failure, which is in accordance with the results from other studies [5,6]. Nevertheless, a recent retrospective study suggests that the formation of circulating anti-donor-HLA antibodies is associated with a decrease of the long-term "homovital" aortic valve allograft performance [20]. Unfortunately, in our series the HLA typing of pulmonary allograft recipients was not available; therefore the results of HLA (mis)match could not be analyzed during this study.

Conclusions. In summary RVOT reconstruction with allografts still is confirmed as a good surgical solution, although progressive allograft failure is noted and careful monitoring of patients is warranted. Further research should reveal a possible general pattern of allograft degeneration, the

clinical importance of immunologic findings, and the best timing for reoperation. The use of aortic allografts is associated with graft dysfunction and should only be used in case of shortage of pulmonary allografts. Our study failed to reveal young donor age and smaller allograft diameter as independent risk factors for accelerated allograft degeneration in younger patients. Nevertheless we advise the use of relatively large allografts in younger patients, in order to postpone reoperation as long as possible.

References

1. Ross DN, Somerville J. Correction of pulmonary atresia with a homograft aortic valve. Lancet 1966;2:1446-7.
2. McGrath LB, Gonzalez-Lavin L, Graf D. Pulmonary homograft implantation for ventricular outflow tract: early phase results. Ann Thorac Surg 1988;45:273-7.
3. Willems TP, Bogers AJJC, Cromme-Dijkhuis AH, et al. Allograft reconstruction of the right ventricular outflow tract. Eur J Cardiothorac Surg 1996;10:609-615.
4. Cleveland DC, Williams WG, Razzouk AJ, et al. Failure of cryopreserved homograft valved conduits in the pulmonary circulation. Circulation 1992;86(Supplement II):151-53.
5. Stark FRCS, Bull C, Stajevic M, Jothi M, Elliott M, de Leval M. Fate of subpulmonary homograft conduits: determinants of late homograft failure. J Thorac Cardiovasc Surg 1998;115:506-16.
6. Niwaya K, Knott-Craig CJ, Lane MM, Chandrasekaren K, Overholt ED, Elkins RC. Cryopreserved homograft valves in the pulmonary position: Risk analysis for intermediate-term failure. J Thorac Cardiovasc Surg 1999;117:141-147.
7. Albert JD, Bishop DA, Fullerton DA, Campbell, Clarke DR. Conduit reconstruction of the right ventricular outflow tract. J Thorac Cardiovasc Surg 1993;106:228-236.
8. Bando K, Danielson GK, Schaff HV, Mair DD, Julsrud PR, Puga FJ. Outcome of pulmonary and aortic homografts for right ventricular outflow tract reconstruction. J Thorac Cardiovasc Surg 1995;109:509-518.
9. Daenen W, Gewillig M. Factors influencing medium-term performance of right-sided cryopreserved homografts. J Heart Valve Dis 1997;6:347-54.
10. Thijsen HJM, Bos E, Konertz W, Van Suylen RJ, De By TMMH. Kryokonservierung humaner Spenderherzklappen in der Herzklappenbank in Rotterdam. Z Herz-, Thorax-, Gefäßchir 1992;6[Suppl 1]:49-55.
11. Edmunds LH, Clark RE, Cohn LH, Grunkemeier GL, Miller DC, Weisel RD. Guidelines for reporting morbidity and mortality after cardiac valvular operations. J Thorac Cardiovasc Surg. 1996;112:707-711.
12. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457-481.
13. Hawkins JA, Bailey WW, Dillon T, Schwartz DC. Midterm results with cryopreserved allograft valved conduits from the right ventricle to the pulmonary arteries. J Thorac Cardiovasc Surg 1992;104:910-16.
14. Livi U, Abdulla A-K, Parker R, Olsen EJ, Path FRC, Ross DN. Viability and morphology of aortic and pulmonary homografts. J Thorac Cardiovasc Surg 1987;93:755-60.
15. Tam RK, Tolan MJ, Zamvar VY, et al. Use of larger sized aortic homograft conduits in right ventricular outflow tract reconstruction. J Heart Valve Dis 1995;4:660-4.
16. Vogt PR, Stallmach T, Niederhauser U, et al. Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infants and adults. Eur J Cardiothorac Surg 1999;15:639-44;discussion 644-5.
17. Rajani B, Mee RB, Ratliff NB. Evidence for rejection of homograft cardiac valves in infants. J Thorac Cardiovasc Surg 1998;115:111-7.
18. Hoekstra FM, Witvliet M, Knoop CY, et al. Immunogenic human leukocyte antigen class II antigens on human cardiac valves induce specific alloantibodies. Ann Thorac Surg 1998;66:2022-6.
19. Oei F, Welters M, Vaessen L, Marquet R, Weimar W, Bogers A. Heart valve dysfunctioning due to cellular rejection in a novel heterotopic transplantation rat model. Transplant International 2000:(in press).

20. Smith JD, Hornick PI, Rasmi N, Rose ML, Yacoub MH. Effect of HLA mismatching and antibody status on "homovital" aortic valve homograft performance. Ann Thorac Surg 1998;66:S212-5.

CHAPTER 3

The presence of immune stimulatory cells in fresh and cryopreserved donor aortic and pulmonary valve allografts

F.B.S. Oei, A.P.A. Stegmann, F. van der Ham , P. E. Zondervan, L.M.B. Vaessen, C.C. Baan, W. Weimar and A.J.J.C. Bogers.

Submitted to The Journal of Heart Valve Disease

Abstract

Background: Heart valve allografts (HVA) used for valve replacement or ventricular outflow tract reconstruction may suffer from structural deterioration due to donor-specific immune responses. The presence of immune stimulatory cells including dendritic cells and activated endothelial cells has not been studied thoroughly in aortic or pulmonary HVA. We analyzed immuno-histochemically the presence and distribution of these cells in both aortic and pulmonary HVA before and after cryopreservation.

Methods: Sixteen aortic and thirteen pulmonary HVA, discarded for implantation due to morphological or technical reasons were obtained from 12 heart-beating and 9 non-heart-beating tissue donors. Aortic and pulmonary HVA were longitudinally dissected into two symmetric sections by splicing of the non-coronary aortic and non-facing pulmonary cusps. Each symmetric half contained one and a half valve cusp attached to the vascular wall. Fresh halves were directly fixed in formaldehyde followed by immuno-histochemical analysis. The corresponding halves of the valves were decontaminated, cryopreserved, stored for at least 3 weeks and thereafter thawed according to the Heart Valve Bank protocol before analysis.

Results: Activated endothelial cells, expressing PECAM-1 (CD31), VCAM-1 and HLA class II molecules covered at least 50% of fresh valvular surfaces. A comprehensive vascular network was found in the myocardial rim and adventitial layer, which was covered entirely by activated endothelial cells. HLA class II positive macrophages (CD68) and T-lymphocytes (CD3) were found scattered in the stroma and subendothelial layer of the valve leaflets. Mononuclear cell clusters were predominantly found in relation to native degenerative foci, more often found in aortic valves. No difference in cellular distribution was observed between the two donor types. Dendritic cells positive for both S100 and CD45 were not found in immuno-double stained sections. Cryopreservation resulted in minor structural alterations in the vascular wall and an increase of cells with pycnotic nuclei and reduction of adhesion molecule expression on endothelial cells.

All fresh and cryopreserved aortic and pulmonary HVA contain abundant HLA class II positive endothelial cells and sparse distribution of mononuclear cells in the luminal and adventitial layers.

Conclusions: Cryopreservation minimally affected the extracellular matrix of HVA and diminished the cellularity of the valve graft, while the HLA class II expression of cells was not abrogated. Aortic valve allografts harbour more mononuclear cells compared to their pulmonary counterparts. The absence of dendritic cells (professional antigen presenting cells) is compensated by the preservation of other cells expressing HLA class II molecules predominantly in the endothelium which may be responsible for initiation of a specific immune response against HVA.

Introduction

The use of human heart valve allografts (HVA) for aortic valve replacements or reconstructions of the right ventricular outflow tract have good clinical results [1, 2]. Like other biological valve prostheses, HVA have distinct clinical advantages over mechanical valves such as the low incidence of thromboembolic complications and the superior hemodynamic properties [3]. However, like other biological prostheses, the majority of HVA may demonstrate structural degeneration in the long run. In pediatric patients, failure of donor HVA appears more rapidly for reasons yet unknown [4]. Although degenerative changes have been observed in donor HVA that were explanted for structural failure, the exact cause for tissue deterioration is not clear [5, 6]. Factors contributing to valve failure may vary and can be categorized into donor and patient related, technical (surgical) and biological related factors [7]. Until now the relevance of immune reactivity in valve tissue destruction has not been clarified while the immunogenicity of HVA is beyond doubt in experimental and clinical studies [8, 9].

The viability of cellular components of the valve may play an important role in the durability of the allograft. The presence of endothelial cells has been suggested to play a role in the graft survival while others suggest matrix fibroblasts to be the more important cellular component for enhancing valve durability [10, 11]. For years the main goal of preservation seemed to preserve the cellularity and structural integrity of the valves. However, enhanced cellularity may result in unimpaired immunogenicity of the valvular tissue.

In organ transplantation, rejection of foreign tissue is dependent on the presence of allogeneic antigens especially those encoded by genes of the major histocompatibilty complex [12]. In humans, these are called the Human Leucocyte Antigens (HLA) and are divided into class I (HLA-ABC) and class II (HLA-DR, DP, DQ) antigens. In the process of immune activation HLA class II antigens play a dominant role as they are needed for antigen presentation [13]. Previous experimental studies have demonstrated that activated donor endothelial cells and dendritic cells are able to stimulate patient T -lymphocytes in a direct way, while patients infiltrating macrophages process donor cells and present donor HLA peptides to patients T-lymphocytes (indirect way) [14,15]. In order to determine the immunogenicity of HVA tissue, the presence of HLA antigens has been studied only in a small series of aortic valve allografts [16]. In the present study we investigated by immunohistochemistry the presence and localization of cells which could have immune stimulatory abilities in both aortic and pulmonary HVA obtained from 2 different types of donors. Additionally, we studied the effect of cryopreservation on the expression of activation induced surface markers on these cells within a single valve cusp.

Materials and Methods

Valve allografts. Sixteen aortic HVA and thirteen pulmonary HVA were obtained from 12 heart beating donors (6 from multi-organ donation procedure and 6 from heart transplant recipients) and 9 non-heart beating tissue donors. Eight pairs of aortic and pulmonary valve allografts were procured from 6 heart beating and 2 non-heart beating donors. Valve allografts included in this study were allografts that were discarded due to native structural defects (e.g. fenestrations, leaflet thickening) or procurement related imperfections (e.g. short truncus, wall or cusp perforations). Additional donor characteristics are summerized in table 1.

Table 1 Donor characteristics

	Aortic valves	**Pulmonary valves**
n	16 (shared pulmonary: 8)	13 (shared aorta: 8)
Gender	Male: 10 / Female: 6	Male: 11 / Female: 2
Age	49.5 (25-64)	47 (25-63)
Donor type	Non Heart Beating: 6 Heart Beating: 10	Non Heart Beating: 5 Heart Beating: 8
Ischemic time	11h (1.5h - 20.75h)	7:75h (6.25h – 20.75h)
Cause of Death	Cardio-myopathy: 5 Myocardial Infarction: 3 Neurological: 8	Cardio-Myopathy: 4 Myocardial Infarction: 2 Neurological: 6 Hepatic Coma : 1
Discard cause	Structural: 13 / Technical: 3	Structural: 4 / Technical: 9

Ischemic time: time between circulation stop and end of cardiectomy during non heart beating donation

After standard aseptic dissection from the heart, the aortic and pulmonary valves were divided into two symmetric halves by longitudinal dissection through the non-coronary cusp and the non-facing cusp, respectively. Each half contains one and a half valve leaflet attached to the vascular root. The right halves were fixed directly in 4% formaldehyde buffered saline for at least 24 hours. The corresponding left halves underwent decontamination, cryopreservation, storage and thawing according to the standard protocol of the Heart Valve Bank Rotterdam. Decontamination of the allografts consist of 6 hours incubation in Medium 199 (Bio-Wittaker, Verviers, Belgium) containing a cocktail of low dose antibiotics including vancomycin 12 μg/ml, flucytosin 30 μg/ml, amikacin 12 μg/ml, ciprofloxacin 3 μg/ml and metronidazol 12 μg/ml in an 37°C incubator. Thereafter, the grafts were transferred into Medium 199 containing 10% dimethyl sulfoxide (DMSO, Sigma, St.Louis, MO) for a controlled-rate freezing at -1°C/min till -80°C and subsequent storage in the vapour phase of liquid nitrogen (-150 to -180°C) at the Heart Valve Bank. After 3 to 6 weeks the valves were rapidly thawed in a 37°C water bath and the DMSO was removed by stepwise dilution in cold Medium 199. Immediately after this standard procedure the valves were fixed in 4% formaldehyde. To optimize antigen preservation the specimens were fixed for a maximum of 36

hours without decalcification followed by further processing by standard formalin fixation and paraffin embedding procedures.

Histological and Immunohistochemical staining. For histological and immunohistochemical evaluation of two corresponding HVA halves, 4μm longitudinal sections were cut from the dissection side of the non-coronary cusp or the non-facing cusp respectively. For histological analysis, sections were stained with hematoxylin-eosin and von Giesson elastin according to standard methods.
Single immunohistochemical staining of the valve sections was performed by using a standard 3-step immunoperoxidase procedure with a set of monoclonal antibodies as primary antisera for detection of specific cellular epitopes (Table 2) [17].

Table 2. Monoclonal antibodies for immunohistochemical single and double staining

Antibodies	Preparation	Dilution	Specificity
JC70A (**CD31**)	pronase	1:60	PECAM-1 (endothelial cells)
rhVCAM-1 (**CD106**, poly) #	citrate-heat	1:75	VCAM-1(endothelial cells)
KP1 (**CD68**)	citrate-heat	1:2000	Monocytes and Macrophages
L26 (**CD20**)	-	1:200	B-lymphocytes
Rabbit- -human(**CD3**, poly)	citrate-heat	1:300	T-Lymphocytes
123c3.D5(**CD56**,NCAM-1)*	citrate-heat	1:25	Natural-Killer cells, Neuroectodermal cells
rabbit- cow(**h-S100**, poly)	-	1:2500	Dendritic,Schwann,, Glial and Ependymal cells
CR3/43 (**h-MHC class II**)	citrate-heat	1:200	HLAntigen class II (HLA-DR, DQ, DP)
2B11 (**CD45**)	-	1:40	Leucocyte Common Antigen
1A4 (**alpha SMA**)	-	1:150	alpha Smouth Muscle Cells and Myofibroblast

*Legend: # by R&D Systems, Abingdon, UK and * by Neomarkers, Fremont, USA. All others by DAKO, Glostrup, Denmark.*

Sections were deparaffinized and the endogenous peroxidase was blocked. If necessary tissue antigens were retrieved by enzymatic digestion (0.1% pronase) or microwave treatment before 30 min. incubation with the primary antibody. Subsequently, the sections were washed with 0.5% PBS/Tween20 and incubated for 10 min with 2% biotinylated goat-anti-multilink (Labvision, Freemont, USA). After washing twice with 0.5% PBS/Tween20 the sections were incubated with 2% of streptavidin-biotin complex (Labvision) for 10 min. followed by incubation with diaminobenzidin.4HCL and 0.3% H_2O_2 for 7 min for final brown precipitation. Slides were subsequently counterstained with Mayer's haematoxylin, dehydrated and mounted. Negative controls included replacement of primary antibodies by phosphate-buffered saline.
To detect dendritic cells, we sequentially immuno-double stained (indirect/indirect) valve sections with CD45 and S100 antibodies [18]. Double staining procedure include incubation for 30 min with specific antisera directed against CD45, followed by washing and 30 min incubation with 2% biotin-labelled multilink secondary antibody in PBS (Biogenex, San Ramon, USA) including 2% human serum and 2% normal gaot serum (Dako Diagnostics, Glostrüp, Denmark). After final incubation

with 2% alkaline-phosphatase labelled streptavidin complex (Biogenex), the detection was done with Fast Blue BB salt (Sigma, Zwijdrecht, Netherlands) which results in blue stained CD45 positive cells. Subsequently, the sections were incubated for 30 min with specific S100 antibody, followed secondary 30 min incubation with 2% goat-anti-rabbit antiserum (Dako). Finally, 30 min incubation with 2% rabbit peroxidase-anti-peroxidase complex (Dako) was followed by staining with 3-amino-9ethylcarbozole (Sigma) and H_2O_2 in 0.02M acetate buffer (pH4.6). This resulted in red staining of S100 positive cells. Cells that are double positive for CD45 and S100 should have dark purple/brown staining. All antibody incubations were performed at room temperature.

Microscopic analysis. Sections were evaluated independently by two investigators (FBSO and APAS) in a blinded fashion. Endothelial lining was carefully examined in the standard hematoxilin-eosin stained section and displayed as the estimated percentage of valve leaflet and vascular wall coverage. The percentage of activated endothelial coverage was scored by examining the CD31 (PECAM-1), CD106 (VCAM-1) and HLA class II positive cells.
The presence of cells expressing HLA class II molecules were evaluated in four different areas of the HVA (vascular wall, lamina fibrosa, lamina spongiosa and lamina ventricularis) and were expressed semi-quantitatively by a graded scale of 0 to 2 based on the the density of the positive cells. The scale is as follows, 0=none, 1=diffuse and 2=diffuse+clusters. A similar scale was used to assign a score for the presence of mononuclear cells (macrophages, B- and T-lymphocytes and NK cells) and the professional antigen presenting dendritic cells (CD45 and S100 positive) in similar valve allograft areas.

Results

Valve structure Thirteen out of 16 (81%) aortic valves showed pre-existing degenerations like fenestrations, fibrosis and sinus wall atheroma, while only 4 out of 13 (31%) pulmonary valve demonstrated structural imperfections. Although the dimensions and thickness appeared to be different between aortic and pulmonary valves, microscopically, they shared the same structures. Both aortic and pulmonary valve leaflets consisted of the usual three layers (lamina fibrosa, spongiosa and ventricularis) each with their specific structures. The vascular wall included in the valve sinus also demonstrated three layers with structural differences (lamina intima, media and adventitia). All fresh aortic and pulmonary specimens showed a normal pattern of elastic tissue in the medial layer consisting of long, uniform and parallel elastine laminae in regular arrangement. After cryopreservation, these elastic laminae became irregular and fragmented in some aortic and pulmonary specimens. The overall cellularity and structure of de valve specimens were not different for the two donor types. Aortic valves showed more HLA class II positive mononuclear cells in the leaflet and vascular wall when compared to pulmonary valves (Fig. 1A+B). Cryopreservation diminished the overall interstitial cellularity in the matrix while the overall collagen structure of the cusps remained intact. In addition, sections of cryopreserved valves include more interstitial cells with pycnotic nuclei when compared to their untreated counterparts.

Endothelial cells Light microscopical analysis of haematoxilin-eosin stained fresh aortic and pulmonary valve sections revealed endothelial cell coverage of the valvular surface for 69% (range 10-95%) and 66% (range 30-95%), respectively. The cryopreserved counterparts did not show significant differences in the endothelial lining. Identification of activated endothelial cells by immunohistochemical staining for PECAM-1 (CD31) (Fig. 4A) and VCAM-1 (CD106) showed valvular surface coverage of approximately 50%(mean, range 10-95%) for both valve types, which decreased significantly after cryopreservation to 35%(mean, range 5-60%, $p<0.05$) (Fig. 2).
More importantly, a confluent layer of endothelial cells, positive for PECAM-1 and VCAM-1, were always present on the luminal surfaces of the vasa vasorum found in the adventitia and outer media layer of the vascular wall (Figure 3A). In contrast to luminal endothelial cells, all micro-vascular endothelium expressed HLA class II and adhesion molecules while cryopreservation appeared not to alter the endothelial lining and the VCAM-1, PECAM-1 and HLA class II expression (Fig. 3B). No difference was found between the aortic and pulmonary valves or donor types. In one aortic and one pulmonary valve from two different donors, a vascular network including activated endothelial cell was found in the hinge area, suggesting the presence of an active vascular blood supply to the valve leaflet.

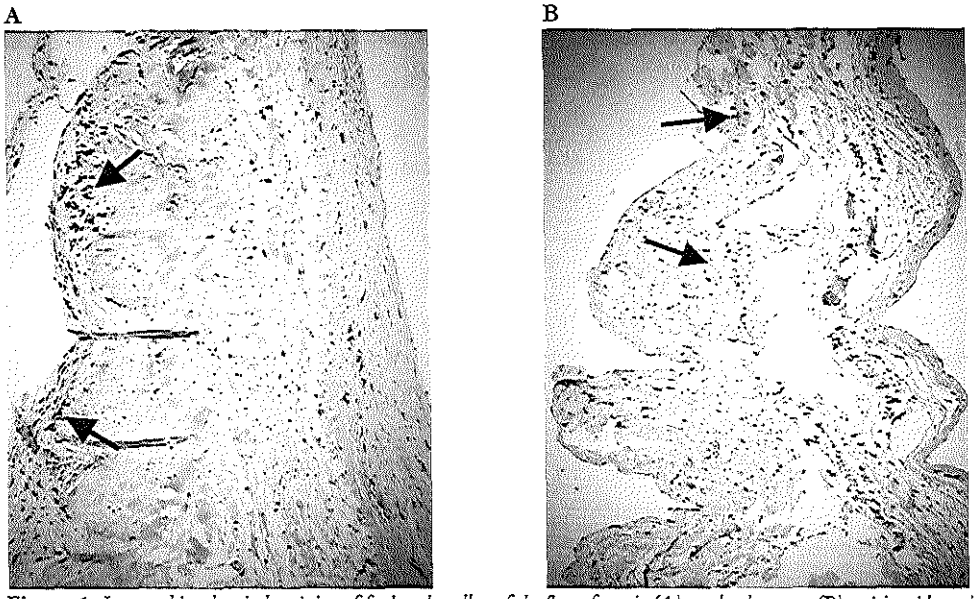


Figure 1. Immunohistochemical staining of fresh valve allograft leaflets of aortic (A) and pulmonary (B) origin with antisera specific for HLA class II. Although the valves were obtained from one single donor the aortic valve contained more HLA calss II positive cells in the leaflets and arterial wall compared to its' pulmonary counterpart. (Original magnification, A and B 50x)

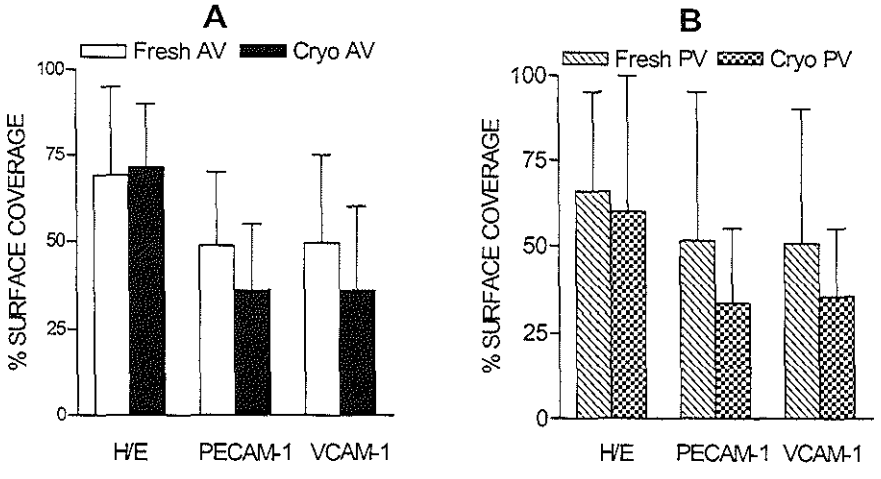


Figure 2. Endothelial lining of HVA were score by analysis of standard Hematoxiline-eosin (H/E) stained and immunohistochemical (PECAM-1 and VCAM-1) stained sections. Bars resemble the median value including the maximum range (lines). Both aortic (A) and pulmonary (B) valves have comparable endothelial coverage when analyzed by morphology (H/E) before and after cryo-treatment. The expression of adhesion molecules (PECAM-1 and VCAM-1) was diminished after cryopreservation in both aortic and pulmonary HVA.

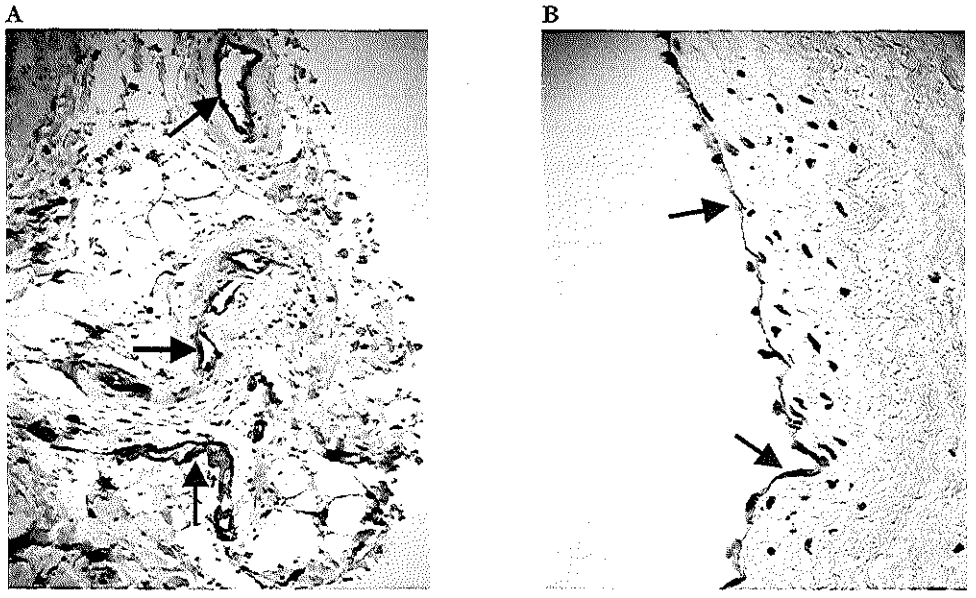


Figure 3. Immunohistochemical staining of HVA with HLA class II antibody demonstrating activated endothelial cells confluent (arrow) with the vasa vasorum of aortic and pulmonary allograft wall (A). Cryopreservation appeared not to decrease the abundant presence of activated endothelial cells in the vasa vasorum. A confluent layer endothelial layer positive stained for PECAM-1 was present on the ventricular side of an aortic leaflet. (B) (Original magnification, A 200x and B 400x)

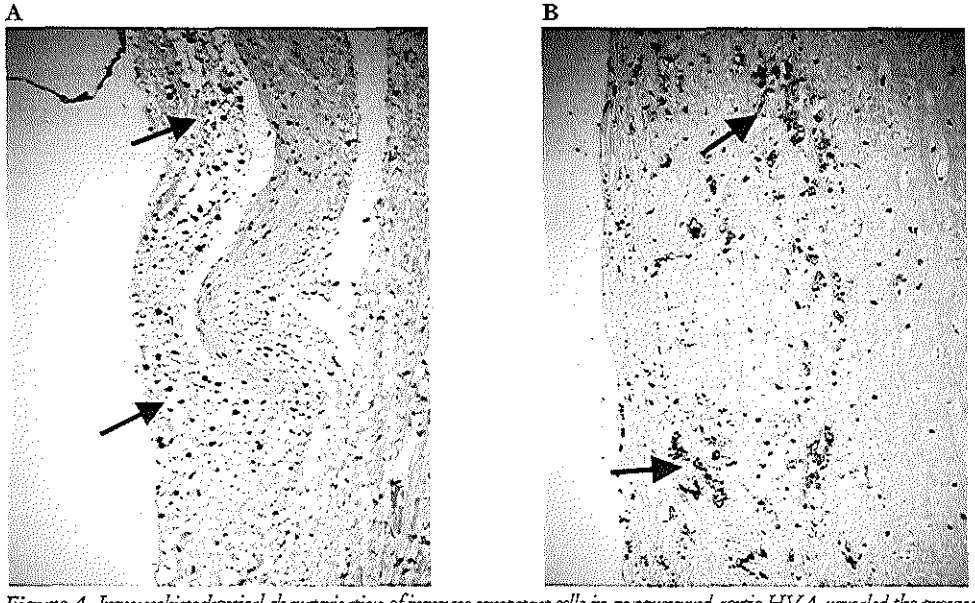


Figure 4. Immunohistochemical characterisation of immune competent cells in cryopreserved aortic HVA revealed the presence of CD3 positive T-lymphocytes clusters (arrows) in the sub-endothelial layer of the ventricular leaflet (A). Clusters of T-cells and CD68 positive macrophages are often seen in reactive intimal thinkening of the arterial wall (B), more frequently seen in aortic HVA. (Original magnification, A and B 200x)

Mononuclear cells In aortic and pulmonary HVA, mononuclear cells positive for CD3 (T-lymphocyte) staining could be found diffusely scattered in all layers of the valve cusp and the vascular wall. Clusters of T lymphocytes were located mainly in the sub-endothelial layer on the ventricular side of the valve leaflet (Fig. 4A), in the spongiosa layer of the hinge region and the vascular wall (intimal and adventitial layer). CD68 positive macrophages were also scattered throughout the leaflet and vascular wall (Fig. 4B). The distribution of macrophages was comparable to that of T lymphocytes. Clusters of T lymphocytes and macrophages were primarily seen in areas demonstrating native degenerative foci like intima thickening of vascular wall and valvular fibrosis or calcification. These pre-existing degenerative changes were present mainly in the aortic valves, resulting in a more prominent occurrence of mononuclear cells in aortic HVA (Fig. 5).
Valve sections immuno-stained with HLA-DR antibodies revealed that these T-lymphocytes and macrophages were strongly positive for HLA class II antigens. After cryopreservation, the amount of mononuclear cells decreased significantly including the cellular expression of HLA class II antigens within the valve leaflets. Very few NK (Natural Killer) cells (CD56+) were present on the ventricular side of the valve leaflet and on the vascular adventitial layer in approximately 50%. In both aortic and pulmonary HVA B-lymphocytes (CD20+) were absent in the leaflets and vascular wall.

Dendritic cells In the lamina spongiosa (central layer) of the valve leaflet and the intimal layer of the vascular wall a large amount of polymorphic S100 positive cells were detected. The distribution of these S100 positive cells was similar for both aorta and pulmonary valves. Comparable to the general cellularity of cryopreserved valves, the amount of S100+ cells also was reduced after cryopreservation. Interestingly, in all sections double stained for S100 protein and Leucocyte Common Antigen (CD45), none of these S100+ cells could be stained with CD45. CD45 staining resulted in identification of the majority of mononuclear cells in valve areas already demonstrated by CD3 and CD68 staining.

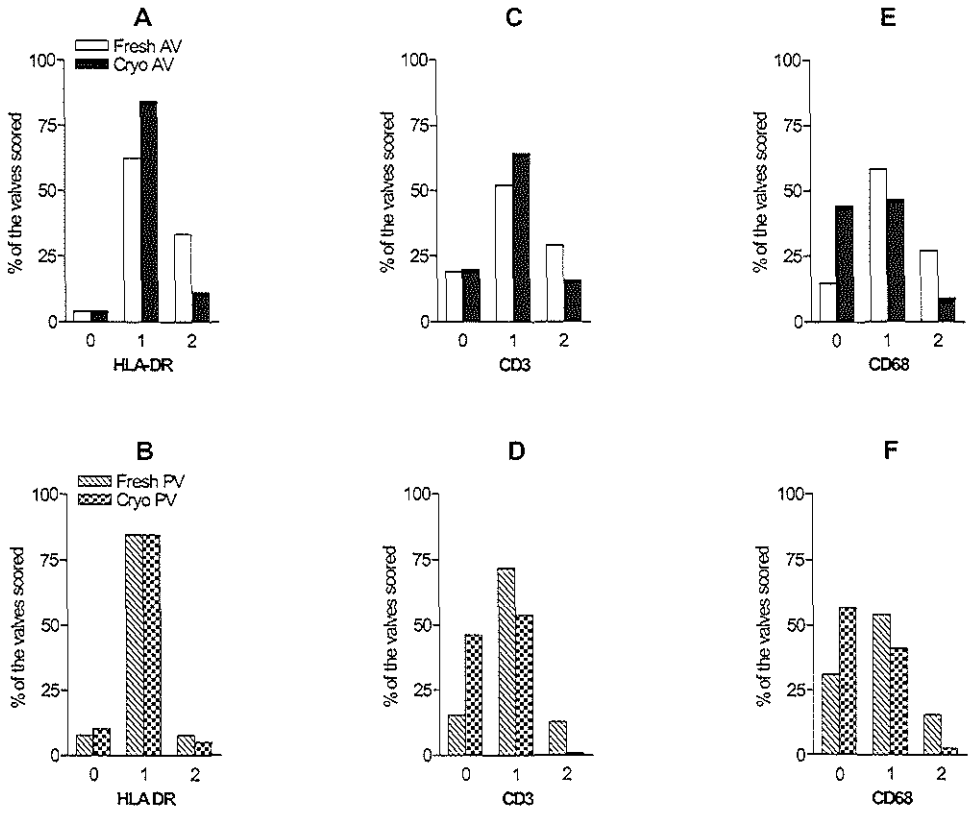


Figure 5. Graphs demonstrating differences in the distribution of HLA-DR (A+B), CD3 (C+D) and CD68 (E+F) positive cells in aortic (AV) and pulmonary valves (PV) leaflets before and after cryopreservation. The presence of positive cells is score semi-quantitatively: 0=none, 1=diffuse, 2=diffuse+focal appearance. Bars represent the percentage of valves provided with a certain score. Cells positive for HLA-DR, CD3 and CD68 are more frequently observed in aortic leaflets compared to their pulmonary counterparts. Cryopreservation reduced the percentage of the total valves with a highest score, which indicate a reduction of the amount of mononuclear cells and cellular HLA class II expression in cryopreserved valve leaflets.

Discussion

In this study, we analyzed the presence and distribution of potential immune stimulatory cells in donor heart valves of aortic and pulmonary origin. By using standard sampling protocols and immuno-histochemical techniques we studied the localization of these cells in one particular valve cusp (non-coronary aortic cusp and non-facing pulmonary cusp) of HVA obtained from two routine donor types. The effect of cryopreservation was studied by comparing treated and untreated symmetric aortic and pulmonary HVA halves. In organ transplantation, the presence of donor-derived dendritic cells in allogeneic tissue has been suggested to play an important role in the immune activation of the recipient, by direct antigen presentation [19]. The question is whether cardiac semilunar valves also contain dendritic cells able to initiate an immune response against the donor HVA. In an immunohistochemical study, Bobryshev and Lord described the presence of vascular dendritic cells in human arterial intima based on single staining with S100 antibody [20]. In the present study, we found S100+ cells abundantly present in the spongiosa and subendothelial lay of aortic and pulmonary valve leaflets, while only a very small fraction of these cells expressed HLA class II. More importantly, none of these S100+ cells stained for Leucocyte Common Antigen (CD45) by immuno-double staining procedure. Based on these results we must conclude that donor valve leaflets, at least in the non-coronary aortic and the non-facing pulmonary cusp, do not contain any substantial amount of dendritic cells. Our suggestion contradicts statements of other researchers based on morphological and single S100 immuno-staining [21,22]. Identification of dendritic cells by morphology or S100 protein expression could overestimate the amount of cells in semilunar valve tissue because both aortic and pulmonary valve leaflets contain distinct nerve fibers and associated nerve terminals which have polyclonal, spindle or stellate shapes [23]. Additionally, all neural structures are positively stained by S100 antibody and consequently could be mis-interpreted as dendritic cells, especially when they share the same area of appearence. Furthermore, valve leaflets also contain smooth muscle cells and fibroblasts, which may have polygonal or spindle shapes and also HLA class II expression when activated [24]. Nevertheless, we can not completely exclude the presence of dendritic cells in human HVA, since we only examined a limited section of one single valve cusp.

Since endothelial cells form the first allogeneic barrier in vascularized organ and tissue transplants they play an important role in the mediation of inflammatory and immunological responses. Human vascular endothelial cells constitutively express HLA class I and can be induced to express class II antigens when activated by interferon-gamma (IFNγ) and tumor necrosis factor (TNF) [25]. The ability of endothelial cells to directly stimulate resting T lymphocytes and thus acting as an antigen presenting cell, seems more and more clear [14,25-26]. Moreover, activated endothelial cells expressing both HLA class I and II molecules can be major targets for CD8 and CD4 cytotoxic T cells [26]. In the present study, all HVA, although in variable degree, showed endothelial coverage

on the luminal surface of the grafts, which was not significantly affected by cryopreservation. The expression of activation markers however was markedly reduced after cryo-treatment, probably due to chemical or mechanical influences during preservation procedure. More importantly, a comprehensive network of small blood vessels, all covered by activated endothelial cells (VCAM-1+ and HLA class II+) was found in the vaso vasorum of the vascular wall in both fresh and cryopreserved HVA. Our findings differ from the results of a study conducted by Yacoub and colleagues, showing that endothelial cells, if present on the valvular surface, were not positive for HLA class II, especially after prolonged storage in 4°C medium [16]. Lupinetti *et al.* described the presence of endothelial cells on valve leaflet and vascular wall surfaces of fresh valves while among cryopreserved specimens obtained from Cryolife, Inc (Marietta, GA), the amount of endothelial cells was significantly reduced [10]. Differences in the donor population, method of analysis and subtle discrepancy in the specific methods of valve processing (e.g. procurement, decontamination, cryopreservation) have been suggested to result in disparate study outcomes [27,28]. Since the majority of activated endothelial cells was localized in the outer layer of the vascular wall it could imply that the host immunological attack starts from the outside and therefore causes a delay in immune activation after implantation of valvular tissue [29,30]. The initiating factor leading to endothelial cell activation, which results in the expression of adhesion molecules like PECAM-1 and VCAM-1 or HLA class II, has been reported to be associated with physiological stress during the peri-mortal or brain death period [31]. Stress induced events including cytokine release and metabolic disturbances have been described to influence donor tissue structures. Novitzky and colleagues reported histological evidences of myocardial degeneration including necrosis and mononuclear cellular infiltration after induction of brain death in a primate model [32]. This phenomenon could be responsible for the presence of T-lymphocytes and macrophages, which are found mainly in the subendothelial layer of the leaflets and vascular wall of valve allografts. On the other hand, Olsson and colleagues found activated T-lymphocytes in normal and non-rheumatic stenotic aortic valves, especially in areas demonstrating calcium deposition. They suggest that apart from a physiological aging process the accumulation of reactive T-cells could have an immunological basis rather than of mechanical origin [33]. Our observation partly supports their theory as we also found clusters of mononuclear cells in relation to tissue fibrosis or calcified spots, which were mostly found in the aortic valves. Based on the differences between pulmonary and aortic valves, we suggest that mechanical stress can influence the influx of mononuclear cells and the initiation or progress of native valve degeneration.

In this study, we showed that the process of cryopreservation reduces the interstitial cellularity of both aortic and pulmonary valve cusps, while the confluent endothelial layer appeared mostly unaffected, except for decreased expression of activation markers. Although there was in some cases fragmentation of the elastine fibers in the vascular wall, the collagen scaffold of the valvular cusp remained intact. Our findings are in agreement with results of comparable studies except for

specimens obtained from non heart beating donors. Different studies reported disintegration of collagen fibers, vacuolization and edema of the cryopreserved leaflets and complete loss of the endothelium from non heart beating donors with increasing ischemic time [22,34]. In this series we did not find structural differences in cryopreserved valves from heart beating or non-heart beating donors. Current dissimilarity in results could be caused by differences in the duration of the processing time (time before freezing), which was relatively short (<50 hours) in the present series.
In conclusion, this immunohistochemical study clarified the cellular basis of the immunogenicity of human aortic and pulmonary semilunar valves. The absence of dendritic cells, as a source for allo-activation via the direct pathway, in both fresh and cryopreserved valves is partially compensated by the presence of activated endothelial cells, macrophages and T lymphocytes. These cells may initiate an allo-response via the indirect pathway. Our observations may have clinical implications as preserved cellularity of valve allografts include expression of strong allogeneic antigens, like HLA class II, that may trigger the immune system of the host by the indirect pathway and consequently serve as immunological targets. If immunological rejection does appear and does result in HVA degeneration, attempts must be made to selectively reduce the immunogeneic load of HVA. However, the sequence of immune activation leading to allograft tissue degeneration remains unclear and should be incorporated in studies identifying factors leading to valve allograft failure. Unlike organ transplantation, no biopsies can be taken from valvular tissue without impairing valve function. Therefore, direct monitoring of potential tissue rejection characterised by local cellular infiltrates and the loss of structural integrity of allogeneic valvular tissue remains impossible. Although animal studies may solve this problem, technical and immunological disparities between clinical and experimental studies may hinder extrapolation to human situation. In organ transplantation, a non invasive in vitro analysis of circulating mononuclear cells can be used as a tool to monitor the immune reactivity of transplant recipients. The increase of a T-lymphocyte fraction with destructive potentials appeared to be associated with histological signs of tissue destruction [35]. Therefore, analysis of circulating mononuclear cells in valve allograft recipients may give us insight in the immunological sequences leading to tissue degeneration and ultimately to valve failure.

References

1. O'Brien MF, Stafford EG, Gardner MAH et al. Allograft aortic valve replacement: longterm follow-up. Ann Thorac Surg 1995;60:S65-70
2. Niwaya K, Knott-Craig CJ, Lane MM, Chandrasekaren K, Overholt ED, Elkins RC. Cryopreserved homograft valves in the pulmonary position: Risk analysis for intermediate-term failure. J Thorac Cardiovasc Surg 1999;117:141-147
3. Kirklin JW, Barratt-Boyes BG, eds. Cardiac Surgery. 2nd ed. New York, NY:Churchill Livingstone; 1993:545-8
4. Baskett RJ, Ross DB, Nanton MA, Murphy DA. Factors in the early failur of cryopreserved homograft pulmonary valves in children: preserved immunogenicity ? J Thorac Cardiovasc Surg 1996;112:1170-9
5. Schoen FJ, Mitchell RN, Jonas RA. Pathological considerations in cryopreserved allograft heart valves. J Heart Valve Dis 1995;4:S72-6
6. Vogt PR, Stallmach T, Niederhauser U et al. Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infacts and adults. Eur J Cardio-thorac Surg 1999;15:639-45
7. McGiffin DC. Invited letter concerning: leaflet viability and the durability of the allograft aortic valve J Thorac Cardiovasc Surg 1994;108:988-90
8. Hoekstra FM, Knoop CJ, Aghai Z et al. Stimulation of immune-competent cells in vitro by human cardiac valve-derived endothelial cells. Ann Thorac Surg 1995;60:S131-4
9. Hogan P, Duplock L, Green M et al. Human aortic valve allograft elicit a donor-specific immune response. J Thorac Cardiovasc Surg 1996;112:1260-7
10. Lupinetti FM, Tsai TT, Kneebone JM, Bove EL. Effect of cryopreservation on the presence of endothelial cells on human valve allografts. J Thorac Cardiovasc Surg 1993;106:912-7
11. Brockbank KGM. Cell viability in fresh, refrigerated and cryopreserved human heart valve leaflets [letter]. Ann Thorac Surg 1990;49:848-9
12. Mason DW, Morris PJ. Effector mechanisms in allograft rejection. Annu Rev Immunol 1986;4:119-45
13. Thorsby E. The role of HLA in T cell activation. Hum Immunol 1984;9:1-7
14. Yun S, Rose ML, Fabre JW. The induction of major histocompatibility complex class II expression is sufficient for the direct activation of human CD4+ T cells by porcine vascular endothelial cells. Transplantation 2000;69:940-4
15. Thery C and Amigorena S. The cell biology of antigen presentation in dendritic cells. Curr Opinon in Immunology 2001;13:45-51
16. Yacoub MH, Suitters A, Khaghani A, Rose M. Localization of major Histocompatibility complex (HLA, ABC, and DR) antigens in aortic homografts. In. Bodnar E, Yacoub M (eds): Biologic and bioprosthetic valves. Proceedings of the third international symposium. New York, York Medical Books 1986, p65-72
17. van der Wal BCH, de Krijger RR, de Herder WW et al. Adult hyperinsulinemic hypoglycemia not caused by an insulinoma: a report of two cases. Virchows Arch 2000;436:481-6
18. Mason DY, Woolston RE: Double immunoenzymatic labelling. In: Bulock GR, Petrusz P (Eds) Techniques in immunohistochemistry. Vol. 1, Academic Press, London, p135
19. Austyn JM and Larsen CP. Migration patterns of dendritic leukocytes. Transplantation 1990;49:1-7
20. Bobryshev YV, Lord RS. S-100 positive cells in human arterial intima and in atherosclerotic leasions. Cardiovascular Research 1995;29:689-96
21. Strutton G, Hogan P, Green M et al. Immunohistochemical analysis of dendritic cells in human aortic valve allografts Abstract nr.40 world symposium on heart valve disease 1999;p92

22. Goffin YAH, Henriques de Gouveia R, Szombathelyi T, Toussaint MJM, Gruys E. Morphological study of homograft valves before and after cryopreservation and after short-term implantation in patients. Cardiovasc Pathol 1997;6:35-42
23. Marron K, Yacoub MH, Polak JM et al. Innervation of human atrioventricular and arterial valves. Circulation 1996;94:368-75
24. Olsson M, Haegerstrand A, Nilsson J. Expresion of HLA-DR antigen and smooyh muscle cell differentiation markers by valvular fibroblasts in degenerative aortic stenosis. JACC 1994:1664-71
25. Prober J, Oroz CG, Rose ML, Savage COS. Can graft endothelial cells initiate a host antigraft immune response? Transplantation 1996;61:343-49
26. Prober JS. Immunobiology of human vascular endothelium. Immunol Res 1999;19:225-32
27. Lange PL, Hopkins RA. Allograft valve banking: techniques and technology. In: Hopkins RA, ed. Cardiac reconstruction with allograft valves. New York:Springer-Verlag. 1989:37-63
28. Mohan R, Feng XJ, Walter P, Herman A. Cryopreserved heart valve allografts can have a normal endothelium. J Thorac Cardiovasc Surg 1994;108:985-7
29. Oei FBS, Welters MJP, Vaessen LMB et al. Induction of cytotoxic T lymphocytes with destructive potential after cardiac valve homograft implantation. J Heart Valve Dis 2000;9:761-8
30. Hoekstra FM, Witvliet M, Knoop CJ. Donor-specific anti-human leukocyte antigen class I antibodies after implantation of cardiac valve allografts. J Heart Lung Transplant 1997;16:570-2
31. Pratschke J, Wilhelm MJ, Kusaka M et al. Brain death and its influence on donor organ quality and outcome after transplantation. Transplantation 1999;67:343-48
32. Novitzky D. Selection and management of cardiac allograft donors. Current Opinon in Organ Transplantation 1998;3:51-61
33. Olsson M, Dalgaard CJ, Haegerstrand A, Rosenqvist M, Ryden L, Nilsson J. Accumulation of T lymphocytes and expression of interleukin-2 receptors in nonrheumatic stenotic aortic valves. JACC 1994;23:1162-70
34. Fischlein T, Schutz A, Uhlig A. Integrity and viability of homograft valves. Eur J Cardio-thorac Surg 1994;8:425-30
35. Vaessen LMB, Daane CR, Maat APA, Balk AHMM, Class FHJ, Weimar W. T-Helper frequencies in peripheral blood reflect donor-directed reactivity in the graft after clinical heart transplantation. Clin Exp Immunol 1999;118:473-479

CHAPTER 4

Increased numbers of circulating donor-specific T helper lymphocytes after human heart valve transplantation

M.J.P. Welters, F.B.S. Oei, L.M.B. Vaessen, A.P.A. Stegmann, A.J.J.C. Bogers and W. Weimar.

Published in Clinical and Experimental Immunology, 2001; 123:1-7

Abstract

Implantation of cryopreserved human donor heart valves for either congenital or acquired cardiac disease has been performed since the last three decades. Although the clinical outcome is good, long-term valve degeneration resulting in dysfunction has been observed. A specific immunological response of the recipient against the allograft has been proposed as one of the factors involved in this process.

Helper T lymphocytes play an important intermediate role in cellular and humoral immune response. Increasing numbers of circulating donor-specific helper T lymphocytes precursors (HTLp) correlate with graft rejection after organ transplantation. To investigate whether cryopreserved human donor heart valves are able to induce a donor-specific T helper response, we monitored the HTLp frequencies (HTLpf) in peripheral blood samples of 13 patients after valve allograft transplantation by use of a limiting dilution assay followed by an interleukin-2 bioassay.

Prior to transplantation, HTLpf specific for donor and third party antigens, showed individual baseline levels. After allografting, the anti-donor frequencies significantly increased in 11 of the 13 patients ($p=0.02$). This was not found for stimulation with third party spleen cells ($p=0.68$), which indicates a donor-specific response. Maximal donor-specific HTLpf were already found at 1-2 months after operation.

Valve allograft transplantation induces an increase in the numbers of donor-specific HTLp in peripheral blood of the patients. Analogous to organ transplantation, these HTLp may play a crucial role in events that lead to valve damage. Therefore, monitoring of HTLp in peripheral blood samples might be informative for donor valve degeneration (rejection) and subsequently valve allograft failure.

Introduction

Since the last 30 years cryopreserved human heart valve allografts have been implanted in patients suffering from congenital or acquired cardiac diseases. In general, the early and midterm clinical outcome is good; however, long-term follow-up studies revealed an increasing incidence of valve allograft failure [1-3]. Various risk factors for valve allograft failure have been described, such as patient and donor characteristics and implantation techniques. Because valve replacement is neither accompanied by blood group or HLA matching between donor and recipient, nor by administration of immunosuppression, an immune response induced by the valve allograft in the recipient has also been suggested to play a role in valve allograft failure. Several studies have demonstrated the presence of immune competent cells like T and B lymphocytes as well as macrophages in explanted valve allografts [4,5] and the ability of valve leaflets to evoke a donor-specific cellular immune response *in vitro* [6]. Induction of antibodies against donor HLA class I and II antigens after valve allografting confirms the activation of both a cellular and humoral immune response [7-9]. An increased prevalence of valve degeneration was found to be associated with HLA antibody formation [10]. However, a causal relationship has not yet been proven between the induction of a cellular immune response and degeneration of heart valve allografts, ultimately requiring retransplantation.

After organ transplantation, frequencies of helper T lymphocyte precursors (HTLpf) and cytotoxic T lymphocyte precursors (CTLpf) in peripheral blood preceded intragraft T cell accumulation and have been correlated with the transplant outcome. Decreased numbers or even lack HTLp were observed in stable engraftment of cardiac and kidney transplants [11,12]. On the other hand, increased donor-reactive HTLpf in peripheral blood of a patient was measured at the time of a rejection episode after cardiac transplantation [13-15]. The prediction of an acute rejection on an individual patient basis was performed by determination of the anti-donor HTLpf, expressed as percentage of the baseline (individual pre-transplantation) value. It was found that a threshold of 150% of the baseline correlated significantly with rejection grade 3, as scored in endomyocardial biopsies according to the criteria of the International Society of Heart and Lung Transplantation [16].

The correlation between numbers of circulating HTLp, as an immunological parameter, and damage of myocytes and infiltration in cardiac transplants was the basis for the underlying study in valve allograft transplantation. In this prospective study, peripheral blood samples of patients after transplantation of cryopreserved human heart valve conduits were monitored for HTLpf, directed specifically against donor HLA antigens, to further investigate the immunological response after valve replacement.

Materials and methods

Patients. Thirteen patients receiving a cryopreserved human heart valve allograft were included in the study after informed consent (Table 1). The median age of the valve allograft recipients was 36 years, ranging from 2 to 55 years. Seven patients received a cryopreserved aortic and six patients a pulmonary heart valve allograft for the first time. This study was approved by the Medical Ethical Committee of the University Hospital Rotterdam and the Medical Faculty of the Erasmus University Rotterdam.

Table 1. Heart valve allograft recipient and donor demographics

	patient		Donor						
Code	gender (M/F)	age (y)	gender (M/F)	age (y)	valve origin	HLA mm[1]	blood transfusion[2]	indication[3]	operation[4]
KP	F	28	F	53	Aortic	2:2:2	1 Ec / 0 Tc	Ai and As	Root replac.
SR	M	55	M	49	Aortic	0:2:1	13 Ec / 3 Tc	Ai	Root replac.
HR	M	42	M	46	Aortic	2:2:2	5 Ec / 1 Tc	Ai	Root replac.
HD	F	34	M	45	Pulmonary	1:1:1	11 Ec / 1 Tc	Ai	Ross proced.
LN	M	52	M	27	Aortic	2:2:1	10 Ec / 0 Tc	Ai	Root replac.
VN	F	30	M	27	Pulmonary	2:2:1	2 Ec / 1 Tc	Fallot	Recon. RVOT
DH	F	36	F	46	Pulmonary	2:1:1	11 Ec / 4 Tc	disc-disc	Recon. RVOT
VG	M	54	F	51	Aortic	1:1:0	0 Ec / 0 Tc	As	Root replac.
JN	M	41	M	55	Pulmonary	2:1:1	6 Ec / 1 Tc	Fallot	Recon. RVOT
HT	M	2	M	16	Pulmonary	2:1:2	1 Ec / 1 Tc	PA &VSD	Recon. RVOT
HS	M	32	M	36	Aortic	2:2:1	7 Ec / 2 Tc	Ai	Root. replac.
LF	M	35	F	38	Aortic	1:2:1	1 Ec / 1 Tc	Ai	Root. replac
ST	M	48	F	61	Aortic	1:2:2	3 Ec / 1 Tc	As	Root. replac

[1]HLA mismatch between valve allograft recipient and donor indicated as mismatch on HLA-A :-B:-DR. nd; not determined. [2]Blood transfusion discriminated between Ec (packed cells; 300 ml per unit) and Tc (platelets; 5 donors per unit). [3]The indication for which valve replacement was required. Ai, aortic valve insufficiency; As, aortic valve stenosis; PA, pulmonary atresia; VSD, ventricular septal defect. [4]Operation performed. RVOT; right ventricular outflow tract.

Valve allografts. Aortic and pulmonary valve allografts, consisting of the vascular root including the three semiluminar valve leaflets, the vascular wall and a limited cardial rim, were obtained from heartbeating donors within 24 hours after death. In the current study, the allografts were received from the Heart Valve Bank Rotterdam, The Netherlands, after allocation by BioImplant Services, Leiden, The Netherlands. Five donors were female and eight were male, the median age was 46 ranging from 16 to 61 years old (Table 1). The donor valves were prepared according to the standard operation procedures of the Heart Valve Bank Rotterdam. After dissection the grafts were sterilized for 6 hours at 37°C in Medium 199 (Bio-Whittaker, Verviers, Belgium) containing a low-concentration of antibiotics (vancomycin 12 μg/ml, flucytosin 30 μg/ml, amikacin 12 μg/ml, metronidazol 12 μg/ml and ciprofloxacin 3 μg/ml). Then, the allografts were

cryopreserved (-1°C/minute) in Medium 199 with 10% dimethyl sulfoxide (DMSO) and stored in the vapour phase of liquid nitrogen (-150 to -180°C) at the Heart Valve Bank Rotterdam. Prior to implantation, the valves were thawed quickly in a 37°C water bath and subsequently the DMSO was removed by stepwise dilutions in cold Medium 199.

Blood sampling. Heparinized peripheral blood samples were taken prior to and at several time points after valve allograft transplantation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Biotech AB, Uppsala, Sweden) density gradient centrifugation and cryopreserved in RPMI 1640 (Bio-Whittaker) containing 10% heat inactivated pooled human serum and 10% dimethylsulphoxide (Sigma, St.Louis, MO, USA) at -140°C until used for functional *in vitro* tests.

Stimulator cells. Donor and third party spleen cells were obtained by homogenization of small pieces of the particular spleen in RPMI 1640 supplemented with 100 U/ml penicillin (Bio-Whittaker), 100 µg/ml streptomycin (Bio-Whittaker) and 10 µg/ml DNase (Sigma).
Subsequently, the homogenate was filtered through a 40 µm cellstrainer (Falcon, Franklin Lakes, NJ), centrifuged over a Ficoll-Hypaque gradient and stored as described for the PBMC.

Limiting dilution assay. Twenty-four replicates of graded numbers of patients' PBMC (responders) were titrated stepwise in seven double dilutions starting from 4 x 10^4 down to 625 cells/ well in 96-well U bottom microtiter plates (Nunclon, Roskilde, Denmark). After 45 Gy irradiation 5 x 10^4 donor or third party spleen cells per well were added as stimulator cells. Third party spleen cells is completely mismatched in HLA type with the donor. Culture medium consisted of RPMI 1640 Dutch Modification (Bio-Whittaker), containing 2 mM L-glutamine (Bio-Whittaker), 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated pooled human serum. Twenty-four wells containing only irradiated stimulator cells served as background control (stimulator block). The plates were centrifuged for 2 min at 200 g and incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2. Then, 100 µl of supernatant per well was transferred to new U-bottom 96-wells plates, in which the interleukin-2 (IL-2) production, as a measure for helper T lymphocyte precursors frequencies (HTLpf), was assessed by use of a bioassay.

IL-2 bioassay. CTLL-2, a cytotoxic T-lymphoblastic cell line derived from a C57/BL6 mouse, is sensitive for murine IL-2 and IL-4 and also for human IL-2, however not for human IL-4 [17]. CTLL-2 cells were continuously cultured and maintained at a concentration of 1- 5 x 10^5 cells per ml in 25 cm^2 flasks (Corning, Costar, Cambridge, MA) with RPMI 1640 (Bio-Whittaker), supplemented with 2 mM L-glutamine, penicillin, streptomycin, 10% bovine calf serum (Hyclone

Labs Inc., Logan, UT), 5 x 10^{-5} M β2-mercaptoethanol (Sigma) and 30 U/ml IL-2 (10% v/v Lymphocult T-LF, Biotest AG, Dreieich, Germany). The culture medium was refreshed for the last time 48 hours before the cells were use in the bioassay. Then, the cells were washed three times in IL-2 free culture medium and resuspended at a concentration of 5 x 10^4 cells/ml. Subsequently, 100 µl of this cell suspension was added to the 100 µl from the LDA culture supernatant and incubated for 24 hours, including a 4-h pulse with ^{3}H-thymidine (spec. act. 5 mCi/ml; Amersham, Aylesbury, UK). As a control, recombinant human IL-2 (Chiron, Amsterdam, The Netherlands) was titrated in double dilutions starting from 200 U/ml. The proliferated CTLL-2 cells, with radioactive thymidine incorporated in their DNA, were harvested with a Basic 96 harvester (Skatron Instruments, Lier, Norway) onto glass fibre filters (LKB-Wallac, Turku, Finland) and analysed by liquid scintillation spectrophotometry (Betaplate 1205, LKB-Wallac) [15].

Statistical analysis. ^{3}H-thymidine incorporation above average background value plus three times the standard deviation was considered positive for IL-2 production. HTLpf (number of IL-2 producing HTLp per million PBMC) and the 95% confidence intervals (CI) were calculated with the maximal likelihood estimation, adapted with a Jacknife method, as described by Strijbosch *et al.* [18]. In this method of calculation, the goodness of fit (GoF) below 12.5 means that the limiting dilution assay fulfils the single-hit kinetic. This is comparable with one straight line in a semilog plot. Differences in HTLpf between various blood sampling time points were tested with Wilcoxon's signed rank test and differences between two defined groups were analysed by Fischer's exact test using InStat software (GraphPad, San Diego, CA). Two-sided p-values <0.05 were considered significant.

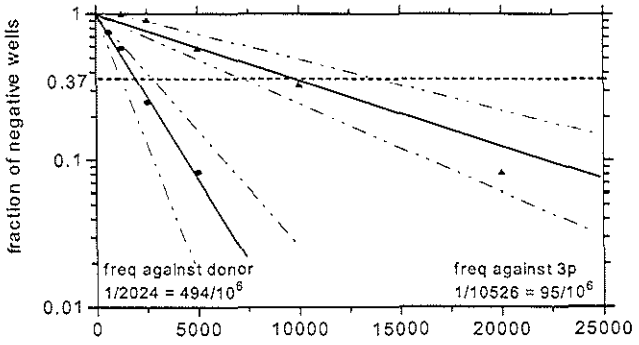


Figure 1. Representative plot of the data from patient HS, 218 days after heart valve allograft transplantation, leading to HTLpf estimation. The estimated frequency against donor (●) was 1/2024 or 494 HTLp/10^6 (GoF=0.6) and against third party (▲) 1/10526 or 95 HTLp/10^6 (GoF=4.3). For each frequency the dashed lines show the 95% confidence limits and the middle (solid) line the estimated HTLpf. The zero term of the Poisson equation predicts that there is on average one HTLp per well when a fraction of 0.37 of the tested wells is negative. The intersection between this 0.37 line and the fitted solid line of fraction negative wells estimates the HTLpf [18].

Results

Longitudinally peripheral blood samples were drawn from each valve allograft recipient. A median number of five blood samples (range 3-6) were taken per patient. In two patients (HT and HR) no blood samples were available between postoperative day 7 and 1 year. In the case of patients HS and LF no blood samples were taken between day 7 and 7 months after implantation of the valve allograft. Currently, the follow-up period of the patients ranges between 1 and 6 years (median 3 years) and all patients are alive and well.

A representative graphic image of the HTLpf estimation of patient HS at post-transplantation day 218 is given in Fig. 1. The frequency against the donor was 494 HTLp/10^6 PBMC and against third party on that particularly time point 95 HTLp/10^6 PBMC. Figure 2 shows for each individual valve allograft recipient the HTLpf against donor and third party HLA antigens in the longitudinally taken peripheral blood samples. In seven patients the pretransplantation frequency for donor and third party was comparable. Before transplantation, in five patients the anti-donor HTLpf was higher than the anti-third party HTLpf, and in one patient (patient HR) the preoperative anti-donor HTLpf was lower than after third party stimulation.

Post-transplantation, in 11 patients the HTLpf against donor cells increased in time ($p=0.02$). A decrease in anti-donor HTLpf was found only in two patients (patient LN and VN). Donor-specific frequencies were maximal at different time points, dependent on the available blood samples. In six of the 11 patients highest HTLpf were found at 1-2 months after the operation. In patient JN, the maximal HTLpf was reached at a later time point, namely at 5 months postoperatively. From patients HS, LF, HR, and HT no earlier blood samples were available, so that the highest levels were found at post-operative month 7, 7, 12 and 55, respectively. In contrast, no such increase was found in third party-specific HTLpf after valve allograft transplantation ($p=0.68$). Over time, 10 of the 13 patients had similar or declining HTLpf against third party antigens. Increasing anti-third party HTLpf were observed only in patient JN, HS and ST.

Comparison of donor and third party stimulation revealed that in 12 of the 13 patients the anti-donor HTLpf was higher than the HTLpf against third party spleen cells. To outline the donor-specificity of the HTL response, the ratio between anti-donor HTLpf and anti-third party HTLpf was calculated and also shown in Fig. 2. A ratio higher than 1 is indicative for a donor-specific response, which was the case in all valve allograft recipients, except for patient HR ($p=0.006$). To determine the relative anti-donor HTLpf, as an indication for rejection, the HTLpf was expressed as percentage of the baseline value, *i.e.* the individual pretransplantation HTLpf against donor HLA [15]. Relative HTLpf of 150% or higher were considered as significant increases, most probably associated with signs of acute rejection. By the use of this method, 11 of the 13 recipients showed an increase in HTLpf against their donor (Table 2). However, in three of these

patients also the relative HTLpf against third party antigens reached levels of 164 to 260%, but this increase was still less pronounced than against donor antigens ($p = 0.005$).

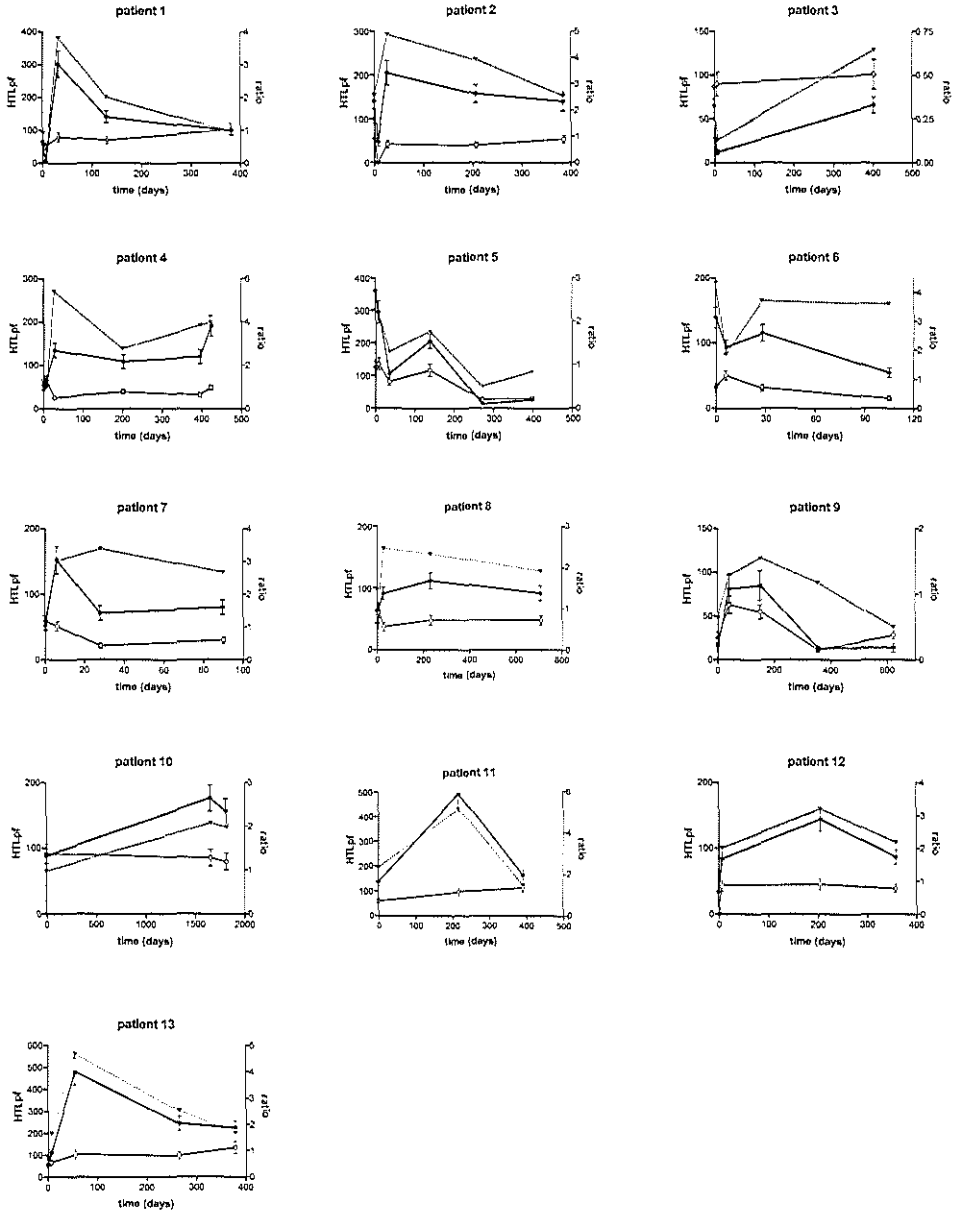


Figure 2. The HTLpf (number of HTLp per million PBMC) ± SD measured against donor (●; solid bold line) and third party (○; solid line) HLA antigens prior to heart valve allograft transplantation (t = 0) and at various time point postoperatively are given on the left y-axis. The HTLpf ratio, anti-donor HTLpf divided by anti-third party HTLpf (▼; dashed line) is depicted on the right y-axis.

Table 2. Individual HTLpf as percentage of the baseline value: measure for rejection

Patient	relative HTLpf									
	anti-donor					anti-third party				
	0	0-3 m	3-6 m	6-12 m	>12 m	0	0-3 m	3-6 m	6-12 m	> 12 m
KP	100	487	227	na	160	100	116	103	na	149
SR	100	150	na	112	101	100	76	na	73	100
HR	100	43	na	na	236	100	103	na	na	116
HD	100	189	na	204	292	100	68	na	58	59
LN	100	55	57	4	7	100	86	93	21	25
VN	100	75	39	na	na	100	127	47	na	na
DH	100	214	154	na	na	100	61	51	na	na
VG	100	124	na	178	146	100	113	na	112	112
JN	100	506	531	81	88	100	252	260	44	112
HT	100	na	na	na	189	100	na	na	na	90
HS	100	na	na	363	120	100	na	na	164	193
LF	100	247	na	424	256	100	100	na	107	95
ST	100	537	na	444	409	100	118	na	135	186

The threshold value of 150% or higher above the baseline value, which correlates with rejection in cardiac transplantation, is given bold. 11/13 patients showed higher relative HTLpf than 150% against donor and only 3/13 patients also against third party antigens (p=0.005). na; peripheral blood samples not available.

Discussion

After cardiac transplantation, accumulation of IL-2 producing T cells in the graft plays an important role in rejection [19]. These rejection episodes are also reflected by increasing numbers of donor-specific HTLp circulating in peripheral blood [11-15]. These findings suggests the presence of activated HTL, that can break through the immunosuppression and produce IL-2, which in its turn mediates a cellular and humoral immune response resulting in myocyte damage and cellular infiltration of the cardiac transplant, signed as rejection. Since human heart valve allograft recipients are not immunosuppressed and these grafts are eventually prone to fail, we studied the ability of these donor valves to activate a cellular immune response by monitoring the HTLpf in peripheral blood samples of 13 patients after valve replacement. Secondly, in concordance to cardiac transplantation, an increase of the relative HTLpf could also be indicative for tissue injury.

The HTLpf measured after valve allograft transplantation were in the same range as those found after cardiac transplantation [15]. Increasing frequencies of HTLp against donor HLA were observed in 11 of the 13 patients ($p=0.02$). The HTLpf in 10 of these 11 patients were higher against donor than against third party antigens (Fig. 2). A proportional increase in anti-third party HTLpf was excluded since the ratio anti-donor / anti-third party HTLpf was calculated. A higher anti-third party than donor-specific HTLpf was observed only in one patient (HR). The immune-competent cells might be more stimulated by these particular third party HLA antigens than by the donor spleen cells. However, it is more likely that the maximal anti-donor HTLpf is missed since no blood samples were available of this patient between one week and 13 months after valve allografting. This could be analogous to the results obtained for patient KP, where these intermediate results were also not present. Nevertheless, the clinical impact of these increasing numbers of donor-specific HTLp after valve transplantation is still not known. Prior to the reached clinical end-points, such as re-transplantation or patient death, echographic analysis is the only method of evaluating the intermediate clinical performance of the donor heart valves in patients. However, as has also been described for cardiac transplants [20-22], this method does not correlate with cardiac graft rejection and valve allograft degeneration.

For cardiac transplant recipients, DeBruyne *et al.* [11,13] and Vaessen *et al.* [14,15] showed that patients with a relative HTLpf of ≥150% of the baseline value, *i.e.* pre-transplantation frequency, were considered at risk for graft rejection. In the present study, 11 of the13 donor valve recipients meet this criterion, and are probably predisposed to valve allograft rejection (see Table 2). Whether valve allograft rejection leads to valve failure, thereby requiring re-transplantation, is still a matter of debate. Unlike for cardiac transplantation, no valve allograft biopsies can be taken and therefore no pathological examination and confirmation of graft rejection can be performed. However, in a heterotopic heart valve transplantation model in rats, we [23] and others [24,25]

have shown a correlation between histological signs of rejection in explanted grafts and valve dysfunction. Preliminary results, using this rat transplantation model, revealed increased donor-reactive HTLpf in the peripheral blood prior to histological signs of rejection and subsequent valve allograft failure.

In conclusion, heart valve allografting induces an increase in donor-specific HTLpf in the peripheral blood. These cells can modulate other immune-competent cells that subsequently can damage the graft (*i.e.* rejection). Since no biopsies can be taken from valve allografts, the analysis of HTLpf might be a (non-invasive) tool to monitor damage. This could be performed in combination with echographic analysis of the valve allograft performance to closely follow the graft outcome. However, further studies are required to determine the assessive or predictive value of HTLpf measurements in peripheral blood samples for rejection and failure of the donor valve.

References

1. Angel WW, Angell JD, Oury JH *et al.* Long-term follow-up of viable frozen aortic homografts. A viable homograft bank. J Thorac Cardiovasc Surg 1987; 93:815-22.
2. O'Brien MF, Stafford EG, Gardner MAH. Allograft aortic valve replacement: Long-term follow-up. Ann Thorac Surg 1995; 60:S65-70.
3. Lund O, Chandrasekaran V, Grocott-Mason R *et al.* Primary aortic valve replacement with allografts over twenty-five years: valve-related and procedure related determinants of outcome. J Thorac Cardiovasc Surg 1999; 117:77-90.
4. Rajani B, Mee RB, Ratliff NB. Evidence for rejection of homograft cardiac valves in infants. J Thorac Cardiovasc Surg 1998; 115:111-7.
5. Vogt PR, Stallmach T, Niederhauser U *et al.* Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infants and adults. Eur J Cardio-thorac Surg 1999; 15:639-45.
6. Hoekstra FME, Knoop CJ, Vaessen LMB *et al.* Donor-specific cellular immune response against human cardiac valve allografts. J Thorac Cardiovasc Surg 1996; 112:281-6.
7. Hoekstra FME, Witvliet M, Knoop CY *et al.* Donor-specific anti-human leukocyte antigen class I antibodies after implantation of cardiac valve allografts. J Heart Lung Transplant 1997; 16:570-2.
8. Hoekstra FME, Witvliet M, Knoop CY *et al.* Immunogenic human leukocyte antigen class II antigens on human cardiac valves induce specific alloantibodies. Ann Thorac Surg 1998; 66:2022-6.
9. Hawkins JA, Breinholt JP, Lambert LM *et al.* Class I and class II anti-HLA antibodies after implantation of cryopreserved allograft material in pediatric patients. J Thorac Cardiovasc Surg 2000; 119:324-30.
10. Smith JD, Hornick PI, Rasmi N *et al.* Effect of HLA mismatching and antibody status on homovital aortic valve homograft performance. Ann Thorac Surg 1998; 66:S212-5.
11. DeBruyne LA, Renlund DG, Bishop DK. Evidence that human cardiac allograft acceptance is associated with a decrease in donor-reactive helper T lymphocytes. Transplantation 1995; 59:778-83.
12. Beik AI, Higgins RM, Lam FT, Morris AG. Clinical significance of selective decline of donor reactive IL-2 producing T lymphocytes after renal transplantation. Transpl Immunol 1997; 5:89-96.
13. DeBruyne LA, Ensley RD, Olsen SL *et al.* Increased frequency of alloantigen-reactive helper T lymphocytes is associated with human cardiac allograft rejection. Transplantation 1993; 56:722-7.
14. Vaessen LMB, Baan CC, Daane CR *et al.* Immunological monitoring in peripheral blood after heart transplantation: frequencies of T-helper cells and precursors of cytotoxic T cells with high avidity for donor antigens correlate with rejection. Transplant Proc 1995; 27:485-7.
15. Vaessen LMB, Daane CR, Maat APWM *et al.* T helper frequencies in peripheral blood reflect donor-directed reactivity in the graft after clinical heart transplantation. Clin Exp Immunol 1999; 118:473-9.
16. Billingham ME, Cary NHB, Hammond ME *et al.* A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection. Heart rejection group. J Heart Lung Transplant 1990; 9:587-93.
17. Deacock S, Schwarer AP, Batchelor JR *et al.* A rapid limiting dilution assay for measuring frequencies of alloreactive interleukin-2 producing T cells in humans. J Immunol Methods 1992; 147:83-92.
18. Strijbosch LWG, Buurman WA, Does RJMM *et al.* Limiting dilution assays: experimental design and statistical analysis. J Immunol Methods 1987; 97:133-40.
19. Baan CC, Holweg CT, Niesters HG *et al.* The nature of acute rejection is associated with development of graft vascular disease after clinical heart transplantation. J Heart Lung Transplant 1998; 17:363-73.

20. Mannaerts HFJ, Balk AHMM, Simoons ML *et al.* Changes in left ventricular function and wall thickness in heart transplant recipients and their relation to acute rejection: an assessment by digitised M mode echocardiography. Br Heart J 1992; 68:356-64.
21. Mannaerts HF, Simoons ML, Balk AH *et al.* Pulsed-wave transmitral doppler do not diagnose moderate acute rejection after heart transplantation. J Heart Lung Transplant 1993; 12:411-21.
22. Valantine HA. Rejection surveillance by doppler echocardiography. J Heart Lung Transplant 1993; 12:422-6.
23. Oei FBS, Welters MJP, Vaessen LMB *et al.* Heart valve dysfunctioning due to cellular rejection in a novel heterotopic transplantation rat model. Transpl Int 2000; 13:S528-31.
24. Moustapha A, Ross DB, Bittira B *et al.* Aortic valve grafts in the rat: evidence for rejection. J Thorac Cardiovasc Surg 1997; 114:891-902.
25. Green MK, Walsh MD, Dare A *et al.* Histological and immunohistochemical responses after aortic valve allografts in the rat. Ann Thorac Surg 1998; 66:S216-2001

CHAPTER 5

Induction of cytotoxic T lymphocytes with destructive potential after cardiac valve allograft implantation

F.B.S Oei, M.J.P. Welters L.M.B. Vaessen, A.P.A. Stegmann, A.J.J.C. Bogers and W. Weimar.

Published in Journal of Heart Valve Disease, 2000; 9: 761-768

Abstract

Clinical and experimental studies have shown that a specific immunological response may play a role in degeneration of human cardiac valves allografts. In heart and corneal transplantation, cytotoxic T lymphocytes (CTL) with high avidity for donor antigens are presumed to be the major effector cells causing graft destruction. We studied the kinetics of these donor-specific CTL precursors (CTLp) and their high-avidity fraction in peripheral blood of patients receiving a cryopreserved valve allograft.

Limiting Dilution Analysis (LDA) was used to enumerate donor-specific CTLp in peripheral blood samples of 15 patients, obtained up to 12 months after valve implantation. Donor-specificity was proven by using donor-HLA mismatched third-party stimulation cells as controls. CD8 monoclonal antibodies were used to distinguish high- and low avidity CTLp.

A significant increase in total donor-specific CTLp among the peripheral blood mononuclear cell population was present seen in 14 out of 15 (93%) patients in the period 3 to 6 months ($p=0.045$) after implantation and remained so for up to 12 months ($p=0.015$). In addition, a significant increase was seen in the fraction of circulating CTLp with high avidity for donor antigens ($p<0.026$) within the first 3 months after implantation.

Implantation of cryopreserved valve allografts increases the number of donor-specific CTLp and their high avidity fraction in the peripheral blood. These cells have the capacity to destruct organ and tissue grafts.

Introduction

Long-term follow-up studies have demonstrated adequate results after implantation of cryopreserved human cardiac valves, especially in adult recipients [1-3]. In contrast to the situation in organ transplantation, the implantation of human cardiac valves is not preceded by blood group (ABO) or Human Leucocyte Antigen (HLA) matching; neither are immunosuppressive medications routinely administered to these valve recipients. However, several studies have given rise to concern about the incidence of graft failure due to immunological reactivity, especially in young recipients [4-6]. Therefore, the clinical impact of the immunogenicity of cardiac valve allografts is still an ongoing matter of debate. Previous studies *in vitro* have outlined the immunogenicity of human cardiac valves [7-9]. In order to analyze the relevance of the immune response in valve degeneration, *ex vivo* studies have been performed on the peripheral blood of valve recipients and valve explants, obtained either during re-operations or at autopsy. These studies demonstrated the presence of donor-specific allo-antibodies in the peripheral blood of valve recipients [10-13]. Additionally, analysis of valve explants has shown the presence of graft infiltrating cytotoxic T lymphocytes specific for donor antigens [9].

In allogeneic heart transplant recipients, the number of cytotoxic T lymphocyte precursors (CTLp) with high-avidity for donor antigens in both peripheral blood and endomyocardial biopsies (EMB) is increased during episodes of acute rejection [14]. The same phenomenon is observed in patients rejecting corneal transplants [15]. Both data suggest that CTLp with high avidity specific for donor antigens are involved in graft destruction. A high-avidity CTL does not require the accessory molecule CD8 for stable binding to HLA-class I molecules and subsequent lysis of the target cell. Therefore, the function of high-avidity CTL cannot be inhibited by the addition of CD8 monoclonal antibodies. We considered whether these potentially destructive cells might also be found in the peripheral blood of patients after implantation of cryopreserved human cardiac valves.

In the present study, we analyzed the kinetics of circulating CTLp and the fraction of CTLp with high avidity in blood samples taken during the first 12 months after valve transplantation. Donor-specific CTLp in serial peripheral blood samples of cryopreserved cardiac valve recipients were enumerated *in vitro* by Limiting Dilution Analysis (LDA). The fraction of CTLp with high-avidity for donor HLA-class I antigens was determined, by addition of CD8 antibodies during the cytotoxic phase of the assay.

Material and Methods

Patients. Fifteen patients (8 males and 7 females, average age 32.3 years ranging from 3 to 57 years) were included in this study after obtaining their consent. The. Four patients received a cryopreserved aortic and 11 patients a cryopreserved pulmonary allogeneic cardiac valve. Four patients received a second allogeneic valve (Table 1). Patient characteristics and the number of HLA-mismatches between donor and recipient for the A, B and DR antigens are summarized in Table 1. All 15 patients received a blood transfusion during the perioperative period while 4 patients received 10 or more units of packed cells because of perioperative blood loss (Table 1). None of the patients had long-term systemic immunosuppressive therapy during the post-operative period. This study was approved by The Medical Ethical Committee of the University Hospital Rotterdam and the Medical Faculty of the Erasmus University Rotterdam.

Table 1 Patient characteristics.

Patient	Sex	Age	Valve origin	Prim/Sec	HLA mismatch	Blood transfusion	Indication	Operation
LN	M	52	Aorta	Primary	2-2-1	Ec 10/ Tc 0	Aortic valve insuff.	Root replac.
DH	F	36	Pulm.	Primary	2-1-1	Ec 11/ Tc 4	Discordant-Discordant	Recon. RVOT
BL	M	57	Aorta	Primary	2-2-1	Ec 6/ Tc 1	Aortic valve insuff.	Root replac.
WS	F	41	Pulm.	Primary	1-2-2	Ec 3/ Tc 1	Fallot	Recon. RVOT
OH	V	34	Pulm.	Primary	1-1-1	Ec 11/ Tc 1	Aortic valve insuff.	Ross. Proced.
RN	M	50	Aorta	Primary	1-1-2	Ec 5/ Tc 0	Aortic valve insuff.	Root replac.
SL	M	24	Pulm.	Primary	2-2-1	Ec 14/ Tc 3	Pulm. atresia and VSD	Recon. RVOT
HK	M	30	Pulm.	Primary	1-2-2	Ec 2/ Tc 0	Tricuspid. atresia	Recon. RVOT
ZD	F	18	Aorta	Primary	2-2-?*	Ec 1/ Tc 0	Aortic valve insuff.	Root replac.
BX	M	16	Pulm.	Second	2-2-1	Ec 2/ Tc 1	Tricuspid. atresia	Recon. RVOT
ZG	F	38	Pulm	Second	2-2-?*	Ec 2/ Tc 1	Fallot	Recon. RVOT
VN	F	30	Pulm.	Primary	2-2-1	Ec 2/ Tc 1	Fallot	Recon. RVOT
SB	F	31	Pulm	Second	1-1-1	Ec 3/ Tc 1	Fallot	Recon. RVOT
HZ	M	3	Pulm.	Primary	1-1-1	Ec 2.5/ Tc 1	Pulm. atresia and VSD	Recon. RVOT
VT	M	25	Pulm.	Second	1-2-1	Ec 8/ Tc 3	Allograft stenosis and insuff.	Ross.proced
Ave.	**M: 8 F: 7**	**32**	**Pulm:11 Aortic:4**	**Prim: 13 Sec.: 2**				

* *In two patients (ZD, ZG) the HLA class II (DR) typing was not available.* **Ec:** *Packed cells: 300 ml per unit* **Tc:** *Platelets: 5 donors per unit* **RVOT:***Right ventricular outflow tract;* **VSD:** *Ventricular septal defect.*

Valve allografts. Aortic and pulmonary roots were obtained from heart-beating and non-heart beating donors within 24 hours after death. Preparation took place under sterile conditions according to the standard operating procedures of the Heart Valve Bank Rotterdam, The Netherlands. After dissection, the grafts were sterilized for 24 hours at 4°C in Medium 199 (Bio-Whittaker, Verviers, Belgium) containing a low-concentration antibiotic solution: 12µg/ml Vancomycin (Eli Lilly), 30µg/ml Flucytosin (Hoechst Pharma), 12µg/ml Amikacin (Bristol-Myers-Squibb), 12µg/ml Metronidazol (Rhone-Poulenc-Rorer) and 3µg/ml Ciprofloxacin (Bayer). Subsequently, the valves were cryopreserved according to the standard cryopreservation protocol of the Heart Valve Bank Rotterdam (-1°C/min.) in Medium 199, containing 10% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA), and stored in the vapor phase of liquid nitrogen (-150 to -180°C). Prior to implantation, the valves were rapidly thawed and the DMSO was diluted stepwise in three 37°C baths containing Medium 199.
Blood sampling. Between 2 to 7 heparin-treated peripheral blood samples were obtained from each patient at different time points up to 12 months after valve implantation. The first blood sample was obtained immediately before surgery. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Biotech AB, Uppsala, Sweden) density gradient centrifugation and slowly frozen in RPMI 1640 medium (Bio-Whittaker) with 10% DMSO (Sigma) and 10% pooled human serum until -80 °C and transferred to storage at -140 °C to await functional *in vitro* tests.

Spleen cell isolation. Spleen cells were obtained by mechanical dissociation of small pieces of spleen obtained from the heart valve donor. Tissue samples were passed through a sieve in RPMI 1640 medium (Bio-Whittaker) containing 10 µg/ml DANSE (Boehringer Mannheim, Mannheim, Germany) to prevent aggregation of cells. After filtration through a 40 µm cellstrainer (Falcon,Franklin Lakes, NJ), the spleen cells were centrifuged over a Ficoll-Paque (Amersham) density gradient, collected, washed and stored at -140 °C.

Limiting Dilution Analysis. Limiting Dilution Analysis was performed in 96-wells U-bottom microtiter culture plates (Nunclon, Roskilde, Denmark)[16]. Responder cells (recipient PBMC) were titrated in seven 1:1 dilution steps starting from $4x10^4$ to 625 cells per well. All dilutions were performed in 24 replicates. To study donor-specificity of the cytotoxic response 2 sets of dilution series were performed. In one series $4x10^4$ irradiated (45 Gy) donor or donor HLA-matched (donor-like) spleen cells were added as stimulator cells. In the other series irradiated (45 Gy) donor HLA-mismatched (third party) spleen cells were used as stimulator cells in equal amounts per well. Each well contained a total volume of 0.2 ml culture medium and the cultures were incubated for 10 days at 37°C in an athmosphere of 5% CO_2. Culture medium consisted of RPMI-1640, Dutch Modification (Bio-Whittaker), supplemented with 2 mM L-glutamine (Gibco

BRL,Scotland,UK), 100 IU/ml penicillin (Bio-Whittaker), 100 μg/ml streptomycin (Bio-Whittaker) and 10% pooled human serum. After day 3, half of the culture medium (100 μgl) was replaced by fresh culture medium, supplemented with 40 U/ml recombinant human interleukin (rIL_2, Proleukin; Chiron BV, Amsterdam, the Netherlands).

Cell Mediated Lympholysis. After 10 days, all 96-well plates were split into two equal volumes (80 μl) and transferred to U-bottom 96-wells plates, with or without CD8 monoclonal antibodies. Subsequently, each well was individually tested for its capacity to lyse $5x10^3$ Europium-labeled PHA blasts of donor(-like) or third party origin. After 4 hours of incubation with Europium-labeled PHA blasts, 20 μl of supernatant was harvested and transferred into 96-well flat-bottom microtiter plates with low background fluorescense (Fluoroimmunoplate, Nunclon)[17]. After addition of 100 μl Enhancement solution (Wallac, Turku, Finland) to each well, the fluorescence of the released Europium was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, Wallac, Finland). Maximum and spontaneous release were defined by incubation of target cells with culture medium in the presence or absence of Triton X-100 detergent (5% v/v solution in 0.01 M TRIS buffer), respectively.

Target cells. Phytohaemagglutinin (PHA; Difco, Detroit, MI)-stimulated T-cell blasts were used as target cells. These cells were obtained by culturing donor(-like) or third-party spleen cells for 7 days in culture medium, supplemented with 10% v/v lymphocult-T (Biotest AG, Dreieich, Germany) and 1% PHA (Difco). After day 7, the T-cell blasts were labeled with Europium (Fluka, Buchs, Switzerland) as reported previously [17]. By using these donor(-like) or third-party PHA-blasts as target cells in the cytotoxic phase of the LDA, only CTLp directed against donor HLA class I antigens (not class II antigens) are determined [18]. Therefore, addition of CD8 monoclonal antibodies alone is sufficient to analyze the avidity of the CTLp.

CD8 inhibition. FK18 (a gift of Dr. F. Koning, Dept. Immunohaematology and Blood Bank, University Hospital Leiden) is a mouse anti-human antibody of the IgG3 subclass, which recognizes the gp32 chain of the CD8 molecule [19]. A concentration of approximately 1 μg IgG3/well of FK18 monoclonal antibody (mAb) obtained from culture supernatant of the Tecnomouse system (Tecnomara-Integra Biosciences, Wallisellen, Switzerland) was used during this study. This concentration totally inhibited the cytotoxic capacity of CD8-dependent CTL clones, but had no effect on cell lysis by CD8-independent CTL clones (17).

Frequency analysis. By analysis of the Poisson distribution relationship between the percentage of cultures that do not show cytotoxicity and the number of responder cells per well, the minimal estimates of CTLp frequencies were calculated. As described by Strijbosch *et al* [20], the

CTLp frequency, expressed as number of CTL per 10^6 PBMC, were calculated with the maximum likelihood estimation, adapted with a jack-knife method. Standard deviation and 95% confidence intervals were also calculated. Therefore, the fraction of donor-specific CTLp having high avidity for donor HLA class I antigens could be calculated using the following formula:

$$\textit{Fraction of high avidity CTLp} = \frac{\textit{freq of CTLp with CD8 mAb}}{\textit{freq of CTLp without CD8 mAb}} \times 100\%$$

Calculation of the high avidity fraction was not performed when the CTLp frequency measured without CD8 monoclonal antibodies was below 10 per 10^6 PBMC, because of unreliable percentages. Statistical analysis were performed with the statistical program INSTAT (GraphPad Sofware, DOS version). For differences in kinetics between groups Wilcoxon signed rank test and Mann-Whitney U-test were used for paired and unpaired evaluation. A two-sided P-value 0.05 was considered significant.

Results

Frequency and kinetic aspects of CTLp in peripheral blood. In the case of 3 patients (D.H., B.X., V.N.) the 12 months follow-up period was incomplete because they stopped visiting the outpatient department at 3 months after valve implantation. The frequency of donor-specific CTLp measured in peripheral blood samples of each patient is shown in Table 2. Although the frequencies varied between the patients, CTLp were present in all pre-transplant blood samples, indicating variable "baseline" immune responses within different individuals. However, in 14 of the 15 (93%) patients the total frequency of CTLp against donor antigens among peripheral blood cells increased during the first 12 months after implantation, when compared with pre-transplantation frequencies. Among those patients, who completed the follow-up period, 2 had reached the maximum frequency at approximately 3 months after implantation (ZD, ZG), while another patient (SB) showed a maximum frequency in the early 1 to 3 weeks post-implantation period. Eight other valve recipients exhibited a maximum frequency in the later period between 6 and 12 months after valve implantation. In one valve recipient (HK) the post-implantation frequency did not exceed the pre-implantation value (Table 2).
The CTLp kinetics of all 15 patients is summarized in Figure 1a. Statistical analysis of the data revealed a significant increase of donor-specific CTLp frequencies in the period 3 to 6 months (median 43, $p=0.046$) and 6 to12 months (median 75.5, $p=0.015$) after implantation, when compared to pre-operative values (median 30). The increase in CTLp frequencies against third party spleen cells was not significant in the 3 to 6 months period (median 30, $p= 0.53$) and the 6 to12 months (median 42, $p=0.07$) (Figure 1b), indicating the donor-specificity of the CTLp response.

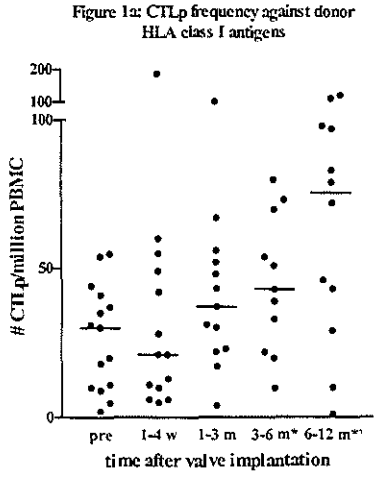
Kinetic aspects of the high-avidity CTLp. Similar to the frequency of total CTLp, the fraction of CTLp with high avidity for donor HLA class I antigens varied between patients (Table 2). In 13 out of 15 cases (86%) the individual profile demonstrated an increase in the CTLp fraction with high avidity within the first 12 months after valve implantation. In two patients (S.B., V.N.) no increase was observed and in one patient (S.B.) the high-avidity fraction of the total CTLp population in pre- and postoperative blood samples were equally high (Table 2). Nevertheless, as shown in Figure 2, the fraction of CTLp with high avidity for donor antigens increased significantly during the period between 1 and 3 months (median 60.5%, $p=0.02$) after implantation compared to pre-operative values (median 21.5%). The maximum was attained in the period between 6 and 12 months after implantation (median 78%, $p=0.006$).

Table 2: Donor-specific CTLp frequencies of 15 cryopreserved valve allograft recipients.

Patient	Pre a / b	1-4 w	1-3 m	3-6 m	6-12 m
LN	31 / 0 (0%)	28 / 0 (0%)	30 / 25 (83%)	33 / nd (-)	72 / 51 (71%)
DH*	18 / 0 (0%)	21 / 2 (10%)	52 / 11 (21%)	39 / 0 (0%)	nd
BL	5 / 0 (-)	5 / 0 (-)	31 / 26 (84%)	20 / 17 (85%)	43 / 41 (95%)
WS	35 / 9 (26%)	42 / 20 (48%)	48 / 27 (56%)	54 / 30 (56%)	83† / 65 (78%)†
OH	54 / 9 (17%)	13 / 12 (92%)	23 / 17 (74%)	nd	97 / 76 (78%)
RN	41 / 19 (46%)	60 / 37 (62%)	67 / 47 (70%)	nd	79 / 75 (95%)
SL	11 / 1 (9%)	11 / 2 (18)	nd	70 / 60 (85%)	111 / 62 (56%)
HK	55 /19 (35%)	10 / 3 (25%)	43 / 12 (28%)	43 / 23 (53%)	29 / 18 (62%)
ZD	2 / 2 (-)	6 / 4 (-)	4 / 0 (-)	22 / 9 (41%)	1 / 1 (-)
BX*	44 / 6 (14%)	55 / 26 (47%)	37 / 9 (24%)	80 / 46 (58%)	nd
ZG	10 / 0 (0%)	21 / 5 (24%)	22 / 6 (27%)	10 / 1 (100%)	10 / 5 (50%)
VN*	37 / 14 (38%)	49 / 8 (16%)	56 / 25 (45%)	73 / 9 (12%)	nd
SB	20 / 19 (95%)	185 / 174 (94%)	102 / 99 (97%)	51 / 47 (92%)	120 / 120 (100%)
HZ	30 /15 (50%)	nd	nd	nd	46 / 42 (91%)
VT	9 / 3 (-)	6 / 1 (-)	17 / 11 (65%)	nd	98 / 69 (70%)
median	**30 / 6 (21.5%)**	**21 / 4.5 (36%)**	**37 / 17 (60.5%)**	**43 / 20 (57%)**	**75.5 / 56.5 (78%)**

*a, CTLp frequency i.e. number of donor-specific CTLp per million PBMC; b, number of high avidity CTLp per million PBMC; c, fraction of high avidity CTLp expressed as percentage of the total CTLp frequency; *, patients with 3 months follow-up; †, blood sample taken at 15.5 months after implantation; nd, not determined; (-), not calculated because the CTLp frequency(a) was below 10 CTLp per million PBMC*

***Figure 1.** a)The kinetics of CTLp frequency directed against donor HLA class I antigens of all 15 patients. A significant increase in the median value is seen 3 to 12 months after implantation compared to the pre-operative value.* * $p=0.046$; ** $p=0.015$*
b)The kinetics of CTLp frequency directed against third party HLA class I antigens of all 15 patients. No significant increase is observed after implantation.



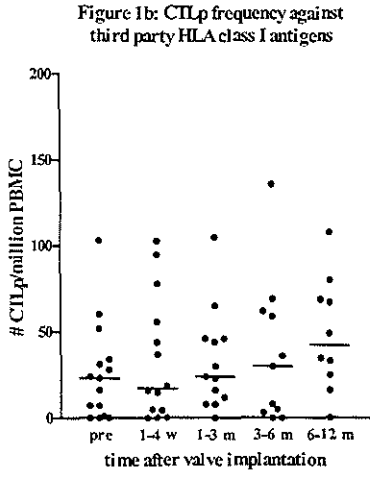
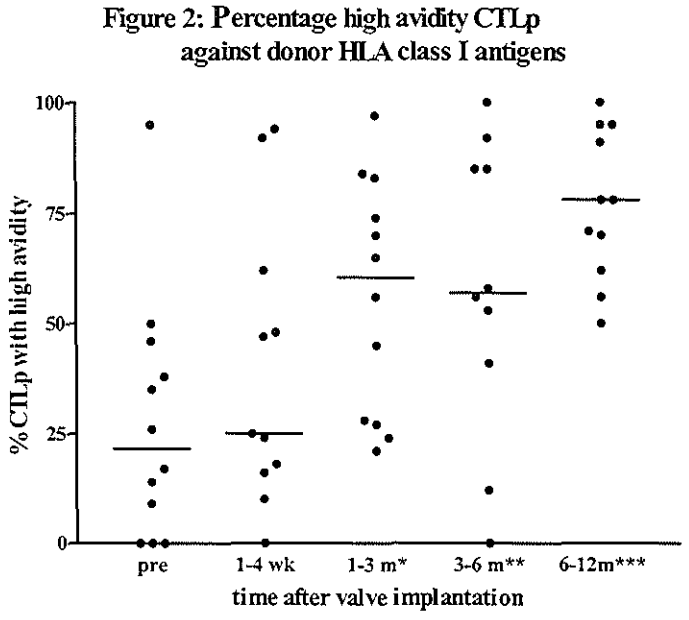


*Figure 2. The kinetics of the CTLp fraction with high avidity for donor HLA class I antigens of all 15 patients. A significant increase was observed from the first 3 months up to12 months after valve implantation in the fraction of high avidity CTLp. * p=0.02; ** p=0.03; *** p=0.006*



Discussion

In the present study, we analyzed the kinetics of circulating donor-specific CTLp and their high avidity fraction in 15 recipients of cryopreserved human cardiac valve allografts. Donor-specific CTLp were detectable in all valve recipients throughout the follow-up period. In 14 patients an increase in the CTLp frequency was observed within 12 months after transplantation, which was not related to postoperative blood transfusion. Interestingly, such an increase in CTLp frequencies was not seen in patients after heart transplantation [18], possibly due to the immunosuppressive agents administered in the latter group. The present study also demonstrates a clear increase in the fraction CTLp with high avidity for donor HLA class I antigens. Data listed in table 2 show that in 86% (13/15) of the patients there was an increase in the CTLp fraction (these cells have CD8-independent cytotoxicity) during the first 12 months after valve implantation. The importance of these donor-specific high avidity CTLp as a marker for acute graft rejection has been outlined in heart as well as corneal transplantation patients [14,15,18,21]. In heart transplantation activated CTL with high avidity for donor antigens and their precursors have been shown to migrate towards the graft shortly before acute rejection becomes histologically evident [14]. In corneal transplant recipients an extensive presence of donor-specific high avidity CTLp and formation of allo-antibodies was observed during rejection, while in non-rejecting patients hardly any of these cells or antibodies were found in the peripheral blood [15,22]. Corneal transplantation is in many aspects similar to cardiac valve transplantation because both techniques involve tissue transplantion without primary HLA matching, blood group matching and immunosuppressive therapies. The increase of the fraction CTLp with high avidity for donor HLA class I antigens in the circulation indicates a cellular immune response induced by implantation of a cryopreserved human valve allograft. Based on the result of cardiac transplantation studies, our data strongly suggests that the CTLp infiltrate into the valve allografts. Furthermore, these findings indirectly demonstrate the presence of cells bearing HLA class I antigens in these cryopreserved valve allografts, which induce the immune response and become target for the immune competent cells. The presence of immunogeneic cells in valve allografts has been described histologically in previous reports [23,24]. As shown in Figure 1a, the increase of the CTLp frequency is significant in the period between 3 and 12 months after implantation. This relatively late onset of the cellular response was also described by Zhao and co-workers [25], who using a rodent model showed a similar, delayed CTLp frequency increase in the recipient animal after heterotopic implantation of fresh aortic valves, while in skin-transplanted animals the increase in CTLp frequency occurred rapidly. There are two possible explanations for the delayed induction of the CTLp response by cryopreserved valve allografts. First, activation of the immune response after organ or tissue transplantation can be established by direct or indirect recognition of allogeneic antigens by immune competent cells of the host.

For direct recognition, antigen presenting cells (APC), like dendritic cells or Langerhans cells, of donor origin are needed to present allogeneic antigen directly, while host APC are responsible for the indirect presentation [26]. Since dendritic cells are myeloid cells, they can be identified by immuno-histochemical double staining with S100 and CD45 (common leucocyte) antibodies. Using this method, Knoop *et al.* demonstrated the presence of many S100 positive cells within both fresh and cryopreserved valve allografts, but none of these cells were positive for CD45 [27]. Therefore, we speculate that the induction of the immune response after valve transplantation may be initiated through the indirect presentation by recipient's APC, loaded with donor HLA-peptides. Activation of the immune response via the indirect pathway is a slower process, because of the time needed by recipient's APC to process and present allogeneic antigens.

A second explanation for the delayed CTLp response might be that valve leaflets and their adjacent intimal layer of the vascular wall may have lost their immunogeneic endothelial cells as a result of their manipulation and cryopreservation [28]. Meanwhile, the tunica adventitia of the vascular wall still contains a comprehensive vascular network including activated endothelial cells which are not destroyed during cryopreservation [27]. These endothelial cells are probably not immediately exposed to immune competent cells after valve implantation, thus resulting in a delayed immune response. Earlier studies regarding activation of the humoral immune response by valve allograft implantation outlined the induction of IgG antibodies against donor HLA class I and II approximately 3 months after valve implantation [10-13], again indicating a delayed immune response and T-helper lymphocyte involvement.

In conclusion, this study confirms that implantation of cryopreserved valve allografts induces a cellular immune response within the recipient. Additionally, the specific increase in the fraction of high-avidity CTLp may indicate immune mediated cellular destruction of allogeneic valvular tissue. However, rejection of a transplanted graft can only to be identified histologically. Unlike organ transplantation, no serial biopsies can be obtained from implanted valve allografts, while analysis of valve allografts explants may not be appropriate because they represent end-stage pathology. Therefore, further studies regarding the consequences of such cellular activity on clinical parameters, indicating structural valve failure [29], are still necessary.

References

1. O'Brien MF, Stafford EG, Gardner MA et al. Allograft aortic valve replacement: Long-term follow-up. Ann Thorac Surg 1995;60:S65-70.
2. Angell WW, Angell JD, Oury JH. Long-term follow-up of viable frozen aortic homografts. J Thorac Cardiovasc Surg 1987;93:815-22.
3. Doty JR, Salazar JD, Liddicoat JR, Flores JH, Doty DB. Aortic valve replacement with cryopreserved aortic allograft: ten-year experience. J Thorac cardiovasc Surg 1998;115:371-9.
4. Clarke DR, Campbell DN, Hayward AR, Bishop DA. Degeneration of aortic valve allografts in young recipients. J Thorac Cardiovasc Surg 1993;105:934-42.
5. Baskett RJ, Ross DB, Nanton MA, Murphy DA. Factors in the early failure of cryopreserved homograft pulmonary valves in children: preserved immunogenicity? J Thorac Cardiovasc Surg 1996;112:1170-9.
6. Salim MA, DiSessa TG, Apert BS. The fate of homograft conduits in children with congenital heart disease: an angiographic study. Ann Thorac Surg 1995;59:67-73.
7. Heslop BF, Wilson SE, Hardy BE. Antigenicity of aortic valve allografts. Ann of Surgery 1973;177:301-6.
8. Ketheesan N, Kearney JN, Ingham E. Assessment of immunogeneicity and viability of homologous human cardiac valves in vitro. J Heart Valve Dis 1996;5:144-7.
9. Hoekstra FM, Knoop CJ, Vaessen LM, Wassenaar C, Jutte N, Weimar W. Donor-specific cellular immune response against human cardiac valve allograft. J Thorac Cardiovasc Surg 1996;112:281-6.
10. Hoekstra FM, Witvliet M, Knoop CJ, Akkersdijk GP, Bogers AJ, Weimar W. Donor-specific anti-human leukocyte antigen class I antibodies after implantation of cardiac valve allogarfts. J Heart Lung Transplantation 1997;16:570-2.
11. Smith JD, Hornick PI, Rasmi N, Rose ML, Yacoub MH. Effect of HLA mismatching and antibody status on "Homovital" aortic valve homograft performance. Ann Thorac Surg 1998;66:212-5.
12. Hogan P., Duplock L., Green M., Smith S., O'Brien M. Human Aortic valve allografts elicit a donor immune response. J Thorac Cardiovasc Surg 1996;112:1260-7.
13. Hoekstra FM, Witvliet M, Weimar W, Claas FH. Immunogenic Human Leukocyte Antigens HLA class II antigens on human cardiac valves induce specific alloantigens. Ann Thorac Surg 1998;66:2022-6.
14. van Emmerik NEM, Vaessen LMB, Balk AHMM, Bos E, Claas FHJ, Weimar W. Progressive accululation of CTL with high avidity for donor antigens during the development of acute cardiac rejection. Transplantation 1996;62:529-36.
15. Roelen DL, van Beelen FPM, van Bree JJ, Claas FHJ. The presence of activated donor HLA class I reactive T lymphocytres (CTLs) is associated with rejection of corneal grafts. Transplantation 1995;59:1039-42.
16. Kaminski E, Hows J, Goldman J, Batchelor R, Optimising a limiting dilution culture system for quentitating frequencies of alloreactive cytotoxic T lymphocyte precursors. Cellular Immunology 1991;137:88-95.
17. Bouma JB, van der Meer-Prins DM, van Rood JJ, Claas FHJ. Determination of cytotoxic T-lymphocyte precursor frequencies using europium labeling as a nonradioactive to labeling with chromium-51. Human Immunology 1992;35:85-92.
18. van Emmerik NEM, Vaessen LMB, Knoop CJ, Weimar W. Kinetics of circulating cytotoxic T lymphocyte precursors that have a high avidity for donor antigens: correlation with the rejection

status of the human cardiac allograft. Transpl Immunol 1998;6:153-60.
19. Koning F, Kordol M, van der Poel J. The influence of workshop monoclonal antibodies on CML, PLT, ADCC, and NK activity. In Reinherz EL., ed. Proceedings of the second international workshop on human leucocyte antigens. Heidelberg: Springer, 1986:189.
20. Strijbosch L, Buurman W, Groenewegen G. Limiting dilution assays. Experimental design and statistical analysis. J Immunol Meth 1987;97:133-40.
21. Ouwehand AJ, Baan CC, Roelen DL. The detection of cytotoxic T cells with high affinity receptors for donor antigens in the transplanted heart as prognostic factor for graft rejection. Transplantation 1993;56:1223-39.
22. Grunnet N, Kristensen T, Kissmeyer-Nielsen F, Ehlers N. Occurrence of lymphocytotoxic lymphocytes and antibodies after corneal transplantation. ACTA Ophtalmologica 1976;54:167
23. Yacoub M, Suitters A, Khaghani A. Localization of Major Histocompatibility Complex (HLA, ABC, and DR) Antigens in aortic homografts. In Bodner E, Yacoub M., eds. Biologic and bioprosthetic valves. Proceedings of the third international symposium. New York: York Medical Books, 1986:65-72.
24. Salomon RN, Friedman GB, Callow AD. Cryopreserved aortic homografts contain viable smooth muscle cells capable of expressing transplantation antigens. J Thorac Cardiovasc Surg 1993;106:1173-80.
25. Zhao XM, Green M, Frazer IF, Hogan P, O'Brien MF. Donor-specific immune response after aortic valve allografting in the rat. Ann Thorac Surg 1994;57:1158-63.
26. Gould DS, Auchincloss H. Direct and indirect recognition: the role of MHC antigens in graft rejection. Immunology Today 1999;20:77-82
27. Knoop CJ, Hoekstra FM, Zondervan P, Bogers AJ, Weimar W. The cellular basis for donor specific humoral and cellular immune responses against cryopreserved human cardiac valve allografts. Abst.nr.17, 6th International Conference on Tissue Banking, Edinburgh, UK, 1997.
28. Lupinetti FM, Tsai TT, Kneebone JM, Bove EL. Effect of cryopreservation on the presence of endothelial cells on human valve allografts. J Thorac Cardiovasc Surg 1993;5:912-7.
29. Edmunds HL, Clark RE, Cohn LH, Grunkemeier GL, Weisel RD. Guidelines for reporting morbidity and mortality after cardiac valvular operations. J Thorac and Cardiovasc Surg 1996;112:708-11.

CHAPTER 6

Circulating donor-specific cytotoxic T lymphocytes with high avidity for donor HLA in pediatric and adult cardiac allograft valved conduit recipients

F.B.S. Oei, M.J.P. Welters, C.J. Knoop, L.M.B. Vaessen, A.P.A. Stegmann, W. Weimar and A.J.J.C Bogers.

Published in European Journal of Cardio-thoracic Surgery, 2000, 18; 466-472

Abstract

Specific immunological responses may be involved in the process of cryopreserved allograft valved conduit (AVC) degeneration, which is more frequently seen in young recipients. Rejection of heart and corneal allografts is preceded by an increase in the fraction of cytotoxic T lymphocytes (CTL) with high-avidity for donor Human Leucocyte Antigens (HLA) circulating in both peripheral blood and the affected graft. These donor-specific high-avidity CTLs are regarded as the destructive cells capable of causing graft damage. To monitor the precursors of these cells (CTLp) in young and adult AVC recipients, *in vitro* quantitative tests were performed on sequentially taken blood samples to quantitate CTLp frequencies and their avidity for donor antigens.

Six children and 9 adults who received a cryopreserved AVC in the period between 1994 and 1997 were included in the study. From these patients, 2 to 6 blood samples were obtained up to 3 years after valve implantation. The number of circulating CTLp present within the peripheral blood mononuclear cell (PBMC) population was determined by Limiting Dilution Analysis (LDA). The fraction of CTLp with high avidity for donor HLA class I was determined by addition of CD8 monoclonal antibodies (mAb) during the cytotoxic phase of the assay. Third party-stimulator cells were used to verify the donor-specificity of the response.

The number of donor-specific CTLp increased significantly in the period 6-12 months after AVC implantation, while third-party-specific CTLp frequencies were not affected. Additionally, we found a significant increase of the high-avidity fraction of CTLp directed against donor antigens, as early as during the first 6 months after AVC implantation. The fraction of high-avidity CTLp remained significantly higher post- compared to pre-implantation, even after 12 months. We observed no significant difference in the kinetics of CTLp frequencies between pediatric and adult AVC recipients.

Implantation of cryopreserved human AVC induces an increase in the total number of circulating CTLp directed against donor HLA class I in both adults and children. The shift towards more destructive high-avidity CTLp in the peripheral blood indicates their potential damaging effect towards the heart valve allograft.

Introduction

Allograft valved conduits (AVC) or heart valve allografts have been used in the last three decades as biological valve prosthesis in the surgical treatment of acquired or congenital heart valve diseases. Several follow-up studies of AVC recipients have revealed good clinical results in the adult population, although in the long run the majority of the grafts showed primary tissue failure [1,2]. In the pediatric population however, the observed early onset of graft failure resulting in allograft replacement is a serious problem [3,4]. A specific immunological response of the recipient against the AVC has been suggested as one of the main causes for allograft degeneration, because AVC implantation is performed without HLA or blood group matching and in the absence of immunosuppressive therapy. Morphological studies of early and late AVC explants have demonstrated the involvement of immune competent cells like T and B lymphocytes as well as macrophages in the valve leaflets and arterial wall of AVC [5,6]. Induction of donor-specific antibodies against HLA class I and II antigens after AVC implantation also confirms activation of cellular and humoral immune responses [7-9]. A causal relationship between an active immune response of the recipient and rejection of the allograft is difficult to prove, since histological confirmation of rejections are not possible.

In heart transplantation, the fraction of precursor cytotoxic T lymphocytes (CTLp) with high-avidity for donor antigens within the total CTLp population, was found to be increased in peripheral blood of the recipients and in myocardial tissue during rejection [10,11]. A similar increase in the fraction of donor-specific high avidity CTL was found in patients rejecting their corneal grafts [12]. Therefore, these CTLp with high avidity for donor antigens are regarded as the major effector cells capable of causing graft damage. Since there is a difference in clinical outcome between pediatric and adult AVC recipients it has been suggested that children are able to mount a more vigorous immune response [5,13].

In an attempt to explain the possible differences in immune response between children and adults, which could lead to destruction of a human AVC, we studied the kinetics of the frequency of CTLp and their avidity in peripheral blood of pediatric and adult AVC recipients.

Materials and Methods

Cardiac allograft valved conduit. Aortic and pulmonary allograft valved conduits (AVC) consisting of the complete arterial root including semilunar valves, arterial wall and a minor muscular rim were obtained from multi-organ donors and non-heart beating donors within 24 hours after circulatory arrest and from so called domino hearts from heart transplant_recipients. Sterile preparation of the AVC was in conformity with the standard operating procedures of the Heart Valve Bank Rotterdam, The Netherlands. After dissection, the AVC were sterilized for 6 hours at 37°C in Medium 199 (Bio-Whittaker, Verviers, Belgium) containing: 12 μg/ml vancomycin (Eli Lilly), 30 μg/ml flucytosin (Hoechst Pharma), 12 μg/ml amikacin (Bristol-Myers-Squibb), 12 μg/ml metronidazol (Rhone-Poulenc-Rorer) and 3 μg/ml ciprofloxacin (Bayer). Subsequently, the allografts were cryopreserved according to the standard cryopreservation protocol of the Heart Valve Bank Rotterdam (-1°C/minute) in Medium 199 with 10% dimethyl-sulfoxide (DMSO, Sigma, St. Louis, MO) and stored in the vapour phase of liquid nitrogen (-150 to -180°C). Before implantation, the allografts are rapidly thawed in a 37°C Medium 199 bath followed by stepwise dilution of the DMSO from the tissue in cold Medium 199.

Patients. Six children (aged under 6 years) and nine adults (>16 years), who received a cryopreserved cardiac AVC between June 1994 and September 1997, were included in the study after informed consent. The average age of the patients in the adult group was 40.2 years (range 25-57), and 9.3 years (range 3-16) in the children group. In the adult group, four patients received a cryopreserved aortic AVC and five patients an allograft of pulmonary origin. In all pediatric recipients, cryopreserved pulmonary AVC were used for reconstruction of the right ventricular outflow tract. Tables 1 and 2 summarize the characteristics of the patients and the indication for AVC implantation. During this study none of the patients were treated with long-term immunosuppressive therapy. The Medical Ethical Committee of the University Hospital Rotterdam and the Medical Faculty of the Erasmus University Rotterdam approved this study.

Peripheral blood mononuclear cells (PBMC) and spleen cells sampling. From each patient, 2 to 6 heparin-treated blood samples were obtained at different time points up to 3 years after AVC implantation. The first blood sample was obtained immediately before surgery. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation and slowly frozen in 10% DMSO (Sigma) and 10% pooled human serum containing RPMI 1640 medium (Bio-Whittaker) until -80°C and transferred to -140°C storage, awaiting for functional *in vitro* tests.

Table 1. Patients characteristics: Adults

Pat.	M/F	Age (y)	Valve origin	Prim/Sec Allograft	Blood transfusion	Indication	Operation
LE	M	52	Aorta	Primary	10 Ec*/ 0 Tc†	Aortic valve insuff.	Root replac.
BY	M	57	Aorta	Primary	6 Ec/ 1 Tc	Aortic valve insuff.	Root replac.
WY	F	41	Pulm.	Primary	3 Ec/ 1 Tc	Fallot	reconstr. RVOT
RY	M	50	Aorta	Primary	5 Ec/ 1 Tc	Aortic valve insuff.	Root replac.
ST	M	24	Pulm.	Primary	14 Ec/ 3 Tc	Pulm.atresia and VSD	reconstr. RVOT
JG	M	41	Pulm.	Primary	6 Ec/ 1 Tc	Fallot	reconstr. RVOT
HR	M	42	Aorta	Primary	5 Ec/ 1 Tc	Aortic valve insuff.	Root replac.
VR	F	30	Pulm.	Primary	2 Ec/ 1 Tc	Fallot	reconstr. RVOT
VE	M	25	Pulm.	Second	10 Ec/ 3 Tc	Allograft sten. and insuff.	Ross procedure
Ave	M: 7	40.2	Pulm: 5	Prim.: 8			
	F: 2		Aortic: 4	Sec.: 1			

**EC: Erythrocytes concentrate 300 ml per unit, †TC: Platelets concentrate 5 donors per unit.*

Table 2. Patients characteristics: Children

Pat.	M/F	Age (y)	Valve Origin	Prim/Sec Allograft	Blood transplusion	Indication	Operation
BU	M	16	Pulm.	Second	2 Ec*/ 1 Tc†	Tricuspid. atresia	Reconst. RVOT
HA	M	3	Pulm	Primary	2.5 Ec/ 1 Tc	Pulm.atresia and VSD	reconstr. RVOT
ML	M	6	Pulm.	Second	1.5 Ec/ 1 Tc	Pulm. atresia and VSD	reconstr. RVOT
KH	V	13	Pulm.	Primary	1.3 Ec/ 0 Tc	Pulm. Stenosis & VSD	reconstr. RVOT
ST	V	6	Pulm.	Primary	3.5 Ec/ 1 Tc	Pulm. Stenosis &VSD	reconstr. RVOT
VM	M	12	Pulm.	Primary	3 Ec / 0 Tc	Truncus Arteriosus	reconstr. RVOT
Ave	M: 4	9.3	Pulm.:6	Prim.: 4			
	F: 2		Aortic:0	Sec.: 2			

** Ec: Erythrocytes concentrate; 300 ml per unit, †Tc: Platelets concentrate; 3 donors per unit*

Spleen cells were obtained by mechanical dissociation of small pieces of spleen from the AVC donor through a sieve in RPMI 1640 medium (Bio-Whittaker) containing 10 µg/ml DNAse (Boehringer Mannheim, Mannheim, Germany) to prevent aggregation of cells. Subsequently, the cell suspension was filtrated through a 40 µm cell strainer (Falcon, Franklin Lakes, NJ) and washed. Thereafter, the spleen cells were centrifuged over a Ficoll-Paque (Amersham) density gradient, collected, washed and stored at -140°C.

Limiting Dilution Analysis. Limiting Dilution cultures were set up in 96-wells U-bottom culture plates (Nunclon, Roskilde, Denmark) [13]. Twenty-four replicates of graded number of responder cells (recipient PBMC) were titrated in 7 steps of 1 : 1 dilutions starting from $4x10^4$ to 625 PBMC per well. To study the specificity of the cytotoxic response two sets of dilution series were performed. In one series $4x10^4$ irradiated (45 Gy) donor or donor HLA-matched (donor-like) spleen cells were

added as stimulator cells. In the other series $4x10^4$ irradiated (45 Gy) donor HLA-mismatched (third-party) spleen cells per well were used as stimulator cells. All wells contained a total volume of 200 µl culture medium, consisting of RPMI 1640 Dutch Modification (Bio-Whittaker), supplemented with 2 mM L-glutamine (Gibco BRL, Scotland, UK), 100 IU/ml penicillin (Bio-Whittaker), 100 µg/ml streptomycin (Bio-Whittaker) and 10% pooled human serum. After 3 days of culturing, 100 µl of the culture medium was refreshed with culture medium supplemented with 40 U/ml recombinant human interleukin 2 (IL_2) (Proleukin; Chiron BV, Amsterdam, The Netherlands).

Cell Mediated Lymphobysis. After 10 days, the microcultures were split in two equal parts (80 µl). One half of the split wells was tested for cytotoxicity in the absence of CD8 monoclonal antibodies (mAb) and the other half was tested in the presence of CD8 mAb. Each well was individually tested for cytolytic activity against $5x10^3$ Europium (Eu-DTPA; Fluka, Buchs, Switzerland and Sigma, St. Louis, MO) labeled target cells [16]. After donor stimulation, T-cell blasts of donor origin were used as targets, whereas third-party stimulated responder PBMC received T-cell blasts of third-party origin. After 4 hours of incubation at 37°C in a humidified atmosphere with 5% CO_2, 20 µl of supernatant was harvested and transferred into 96-well flat-bottom microtiter plates with low background fluorescence (Fluoroimmunoplate, Nunclon). Subsequently, 100 µl Enhancement solution (Wallac, Turku, Finland) was added to each well and the fluorescence of the released Europium was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, Wallac, Finland). Fluorescence was expressed in counts/s. As a control for each target cell, spontaneous lysis (target cells + culture medium) and maximum lysis (target cells + 1% Triton X-100) was determined. The fraction of CTLp with high avidity for donor or third-party HLA class I antigens was calculated using the formula:

$$\%\text{CTLp with high avidity} = 100\% \times \frac{\text{CTLp frequency with CD8}}{\text{CTLp frequency without CD8}}$$

Target cells. T-cell blasts were obtained by culturing donor or third-party spleen cells for 7 days in culture medium supplemented with 1% phytohaemagglutinin (PHA; Difco Laboratories, Detroit, MI,) and 10% v/v lymphocult-T (Biotest AG, Dreieich, Germany). Then, the T-cell blasts were labeled with Europium as reported previously by Bouma *et al* [16] These T-cell blasts can be used to determine donor HLA class I (but not class II) directed cytotoxicity [10].

CD8 inhibition. FK18 (gift of Dr. F. Koning, Dept. Immunohaematology and Blood Bank, University Hospital Leiden, The Netherlands) is a mouse anti-human antibody of the IgG3 subclass, which recognizes the gp32 chain of the CD8 molecule [17]. In this study, a concentration of approximately 1μg IgG3/well of FK18 mAb obtained from culture supernatant of the Tecnomouse system (Tecnomara-Integra Bioscience, Wallisellen, Switzerland) was used. This concentration inhibited the cytotoxic capacity of CD8-dependent CTLp clones but had no effect on cell lysis by CD8-independent CTLp clones [10].

Statistical analysis. Experimental wells were scored positive, if the counts of the well exceeded the mean counts/s + 3x SD of the 24 wells in which only stimulator cells were present. For each responder cell concentration the number of negative wells were determined and used to calculate the frequency with a statistical program designed by Strijbosch *et al* [18] The CTLp frequency (expressed as the number of CTLp per 10^6 PBMC) and the 95% confidence interval (CI) were calculated by the Jackknife procedure for maximal likelihood. The post-operative follow up is divided in three periods: 1-6 months, 6-12 months and >12 months. When a time period includes multiple blood samples of one patient the average of the analyzed frequencies was calculated. The significance of differences in CTLp frequencies or the percentage of high-avidity CTLp against donor or third-party antigens at different periods after AVC implantation was analyzed by the Mann-Whitney U-test. The paired Wilcoxon signed rank test was used to evaluate the frequency kinetics in both children and adult group. Two-sided p-values of 0.05 or less were considered significant.

Results

Follow-up of valve allograft recipients. From 1 adult (V.N.) and 1 pediatric (V.M.) AVC recipient the follow-up period was less than 6 months, because they stopped visiting the outpatient department at 3.5 months (Table 3). The mean follow-up period of the adult patients was 19.8 months (range 3.5-36.5m) and 19.0 months (range 4.7-35.5m) for the pediatric group. From two pediatric patients (H.Z., B.X.) we did not receive blood samples in the period 1-6 months and 6-12 months, respectively. From one adult patient (HR) the 1-6 months blood sample was not available. At the time of completion of this study all patients are still alive.

CTLp frequency in peripheral blood. The number of donor-specific CTLp per million PBMC, the CTLp frequency, of all 15 patients increased in time compared to the pre-operative value. This was not related to the amount of blood transfusions each patient received (Table 1). In the period 6-12 months after AVC implantation the CTLp frequencies were maximal and significantly higher than before implantation ($p=0.01$; Table 3). In 12 patients the pre-implantation CTLp frequencies were below 50, and for three AVC recipients (H.R., S.T. and V.M.), they exceeded 100 CTLp per million PBMC. No significant increase of CTLp frequencies against third-party antigens was observed at 1-6 ($p=0.82$), 6-12 ($p=0.41$) and >12 ($p=0.89$) months after implantation (Figure 1b), indicating the donor-specificity of the CTLp response.

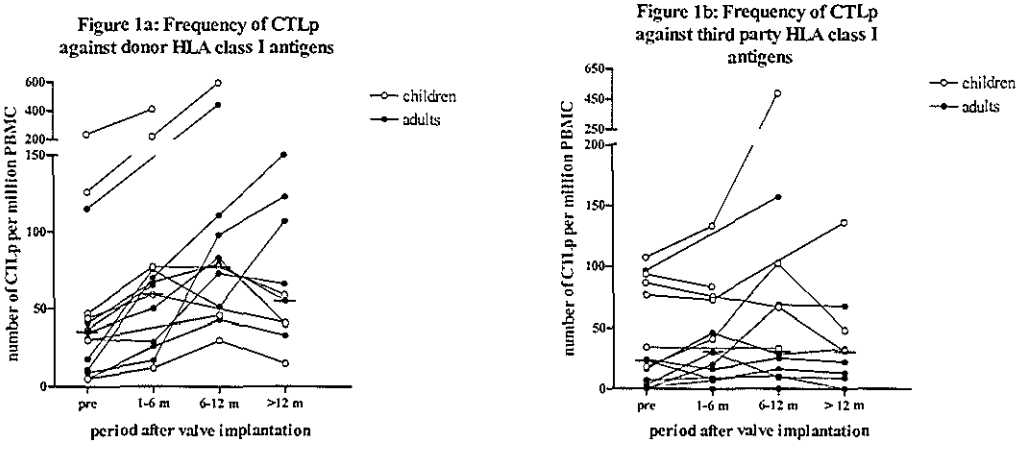


Figure 1.

a) Kinetics of the CTLp frequency directed against donor HLA class I antigens of 6 pediatric and 9 adult patients. Horizontal bars resemble median value of all 15 patients. The increase of CTLp frequency in all 15 patients was significant during 6 to 12 months after valve implantation ($p=0.01$).

b) Kinetics of CTLp frequency directed against donor mismatched HLA class I antigens (third party) of children and adult recipients. Horizontal bars resemble median value of all 15 patients. No significant increase in frequency was observed after valve implantation.

Fraction of CD8-independent (high avidity) CTLp. Similar to the total frequency of donor-specific CTLp, the fraction of CTLp with high avidity for donor antigens varied between the 15 patients (Figure 2). In 13 out of 15 AVC recipients (86%) the percentage of donor-specific high avidity CTLp post-operatively was higher compared to pre-implantation values. It should be noted that from the two patients (V.N., V.M.), in whom the fraction high- avidity CTLp remained unchanged, the follow-up period was incomplete (Table 3). The highest levels were observed at 6-12 months ($p < 0.0001$, Table 3). After the first 12 months the fraction high-avidity CTLp declined in five patients (Table 3). Nevertheless, the median value remained significantly higher compared to the pre-operative fraction ($p=0.01$; Table 3).

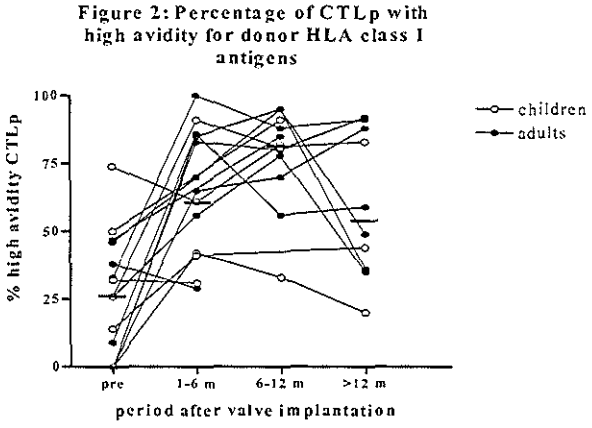


Figure 2.
The kinetics of CD8-independent (high avidity) CTLp fraction specific for donor HLA class I antigens of 6 pediatric and 9 adult patients. Horizontal bars resemble median value of all 15 patients. The post-implantation increase of the donor-specific high avidity fraction of CTLp of all patients was significant in patients compared to pre-implantation.

Comparison of the frequency kinetics between pediatric and adult patients. The paired Wilcoxon signed rank test was used to determine the frequency kinetics of each patient group. In both pediatric and adult AVC recipients the CTLp frequencies increased in time (Figure 1a). Statistical analysis of the pre- and highest post-implantation values revealed *p*-values of 0.03 and 0.004 for children and adults, respectively. The kinetics in pediatric AVC recipients was comparable to that in adults, because the absolute increase of the CTLp frequency after AVC implantation (*i.e.* ΔCTLp frequency) in children and adults was not significantly different during the three time periods, i.e. first and second 6 months and after the first year (Mann-Whitney U-test; $p=0.44$, $p=0.21$, $p=0.07$ *respectively*).
In the adult group the median fraction donor-specific high avidity CTLp increased significantly ($p=0.008$), whereas in the children group the increase is almost significant ($p=0.06$) due to the small numbers of patients.

Comparison of the delta (Δ) increases of the fraction high-avidity CTLp in children versus adults, using the Mann-Whitney U-test, showed no significant differences in the increase during the three observation periods ($p=0.13$, $p=0.08$, $p=0.80$).

Table 3. Donor-specific CTLp frequency analysis of pediatric and adult cryopreserved valve allograft recipients

Patient	Pre a / b (%)	1- 6 m (%)	6- 12 m (%)	> 12 m (%)
LE	31 / 0%	29 / 83%	73 / 80%	66 / 92%
BY	5 / -	26 / 85%	43 / 95%	33 / 36%
WY	35 / 26%	50/ 56%	83/ 78%	40/ 35%
RY	41 / 46%	67 / 70%	79 / 95%	55 / 49%
ST	11 / 9 %	70 / 86%	111 / 56%	155 / 59%
JG	18 / 33%	75 / 33%	51 / 100%	107 / 88%
HR	115 / 47%	nd	445 / 85%	nd
VR*	37 / 38%	65 / 29%	nd	nd
VE	9 / -	17 / 65%	98 / 70%	123 / 88%
BU†	44 / 14%	59 / 41%	nd	41 / 44%
HA†	30 / 50%	nd	46 / 91%	nd
ML†	5 / -	12 / 42%	30 / 33%	15 / 20%
KH	7 / 26%	77 / 91%	77 / 81%	59 / 83%
ST†	126 / 74%	220 / 61%	596 / 82%	nd
VM*†	236 / 32%	419 / 31%	nd	nd
Median	**30.5 / 32.5%**	**51 / 62.5%**	**75 / 80.5%**	**66 / 59%**

*a, CTLp frequency i.e.number of donor-specific CTLp per million PBMC; b, fraction of high avidity CTLp expressedas percentage of the total CTLp frequency; *, patients with 3 months follow-up; †,pediatric patient; nd, not determined; (-), not calculated because the] CTLp frequency (a) was below 10 CTLp per million PBMC.*

Discussion

Since the introduction of cardiac AVCs as biological substitutes in valve replacement surgery, the risk of tissue degeneration due to immunological reactions has been a matter of debate. Viability preservation of AVCs has been suggested to improve durability [19], while others promote preservation of the non-vital collagenous network as an important criteria for prolonged allograft durability [5, 20]. Cryopreservation represents a cell– and tissue protective preservation method, which preserves the cellular viability as well as the stromal structure. The question regarding the loss of the endothelium expressing HLA class I and II molecules remains unclear, since conflicting results have been published [21, 22] In this clinical study, we could not assess the presence of viable endothelial cells in the pre-implantation AVCs. However, fresh as well as cryopreserved human aortic valve fragments or valve-derived endothelial cells were able to stimulate allogeneic T-cells *in vitro* [23]. Apart from vascular endothelial cells, dendritic cells have been identified in cryopreserved human AVC's, which are capable of presenting foreign HLA class I and II antigens to recipient T-cells [24]. Additionally, fibroblasts, smooth muscle cells and dendritic cells, bearing HLA class I molecules have been found to be embedded in the matrix of heart valve leaflets [19, 25]. These cells can serve as antigen source to initiate an immune response by the indirect pathway [26]. The results of the present study demonstrate an overall increase of the total CTLp frequency in peripheral blood of all 15 patients during the first year after cryopreserved AVC implantation (Figure 1a). These data again support the assumption that cryopreserved AVCs contain viable cells expressing HLA class I and II molecules, able to induce a donor-specific cellular and humoral immune response in the recipient [7, 9].

In heart transplant recipients, not the absolute increase in the number of circulating donor-specific CTLp but the increase of CD8-independent (high-avidity) CTLp within the CTLp population is associated with graft rejection [10]. It has been suggested that the strict immune-suppressive regimen of heart transplant recipients inhibits CTLp numbers, while destructive high-avidity CTLp that can break through the immune-suppressive barrier cause graft rejection. A similar increase of the donor-specific high-avidity CTLp fraction was seen in patients rejecting their corneal transplant graft, while in non-rejecting patients, only CD8-dependent (low avidity) CTLp were found in the peripheral blood [12]. The increase of the donor-specific high avidity CTLp fraction in 13 out of 15 valve recipients in this study is consistent with these data and may imply a process of cell-mediated injury of the AVC.

Infiltration of AVCs by immune competent cells like T and B lymphocytes and macrophages, similar to rejection, was demonstrated by different groups using *in vivo* animal studies [27, 28]. However, contradictory reports have been published over the years concerning descriptive morphological studies of clinical valve allograft explants. Mitchell and associates described only sparse mononuclear inflammatory cell infiltration in 20 explanted cryopreseved valve allografts,

which all appeared non-viable without endothelial or connective tissue cells. They suggested that immune responsiveness has no impact on allograft degeneration [20]. On the other hand, Rajani and colleagues demonstrated lymphocytic infiltration in valve leaflets of five valve allografts explanted due to structural failure within less than 8 months after implantation [6]. Others reported identical infiltration of inflammatory cells in cryopreserved pulmonary valve allograft explants, while the aortic valve explants were free from any cellular component [5]. Finally, Hoekstra *et al* demonstrated donor-specific cytotoxicity of infiltrating lymphocytes found in explanted AVC [29]. Also , reviewing these studies, severe structural failure associated with infiltration of immune competent cells is more frequently seen in pediatric patients, in whom pulmonary AVC s are predominantly implanted right-sided. The question remains whether the higher incidence of structural failure is due to increased immune reactivity of the pediatric recipient, to intrinsic differences between cryopreserved pulmonary and aortic AVCs or to surgical_techniques applied. The hypothesis of a more potent immune response in young children is not yet established and is not supported by this study. However, a recent study regarding pediatric renal transplantation demonstrated more rejection episodes and a greater irreversibility of rejection in younger recipients, supporting the hypothesis of an increased immune responsiveness [30]. Ettenger proposed higher functional indices of immune competent cells in children less than 5 years old as a cause for higher immune reactivity [31]. In our study, we have demonstrated a comparable increase in both the number of circulating CTLp and its high-avidity fraction within pediatric and adult patients. The small number of pediatric patients and the lack of patients, who reached defined end of analysis (*i.e.*, re-operation or death due to severe valve degeneration) are both definite limitations of this single-center study. Apart from expanding the study-population, further studies concerning the clinical consequences of such immunological activity have to be performed using relevant clinical parameters for structural valve failure [32].

In conclusion, cryopreserved cardiac AVCs are able to promote an increase in the number of circulating CTLp specific for donor HLA class I antigens. The preferential expansion of the high avidity fraction of CTLp that we have found, may reflect an ongoing process of cell-mediated destruction of the valve allograft tissue *in situ*, which ultimately may lead to or contribute to primary structural failure. We observed no differences in the kinetics of CTLp frequency between pediatric and adult recipients.

References

1. O'Brien MF, Stafford EG, Gardner MA. Allograft aortic valve replacement: Long-term follow-up. Ann Thorac Surg 1995; 60:S65-70
2. Lund O, Chandrasekaran V, Grocott-Mason R, Elwidaa H, Mazhar R, Khaghani A, Mitchell A, Ilsley C, Yacoub MH. Primary aortic valve replacement with allografts over twenty-five years: valve- related and procedure-related determinants of outcome. J Thorac Cardiovasc Surg. 1999;117:77-90
3. Baskett RJ, Ross DB, Nanton MA, Murphy DA. Factors in the early failure of cryopreserved homograft pulmonary valves in children: preserved immunogenicity? J Thorac Cardiovasc Surg 1996;112:1170-1179
4. Salim MA, DiSessa TG, Apert BS. The fate of homograft conduits in children with congenital heart disease: an angiographic study. Ann Thorac Surg 1995;59:67-73
5. Vogt PR, Stallmach T, Niederhauser U, Schneider J, Zund G, Lachat M, Kunzli A, Turina MI. Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infants and aults. Eur J Cardio-thorac Surg 1999;15:639-45
6. Rajani B, Mee RB, Ratliff NB. Evidence for rejection of homograft cardiac valves in infants. J Thorac Cardiovasc Surg 1998;115:111-7
7. Hogan P., Duplock L., Green M., Smith S., O'Brien M. Human Aortic valve allografts elicit a donor immune response. J Thorac Cardiovasc Surg 1996;112:1260-67
8. Smith JD, Hornick PI, Rasmi N, Rose ML, Yacoub MH. Effect of HLA mismatching and antibody status on "Homovital" aortic valve homograft performance. Ann Thorac Surg 1998;66:212-215
9. Shaddy RE, Hunter DD, Osborn KA, Lambert LM, Minich LL, Hawkins JA, McGough EC, Fuller TC. Prospective analysis of HLA immunogeneicity of cryopreserved valve allografts used in pediatric heart surgery. Circulation 1996;94:1063-67
10. van Emmerik NEM, Vaessen LMB, Knoop CJ, Weimar W. Kinetics of circulating cytotoxic T lymphocyte precursors that have a high avidity for donor antigens: correlation with the rejection status of the human cardiac allograft. Transpl Immunol 1998;6:153-60
11. Vaessen LMB, Baan CC, Claas FHJ, Weimar W. Differential avidity and cyclosporine sensitivity of graft-infiltrating, donor-specific CTL and their precursors. Relevance for clinical cardiac graft rejection. Transplantation 1994;57:1051-9
12. Roelen DL, van Beelen FPM, van Bree JJ, Claas FHJ. The presence of activated donor HLA class I reactive T lymphocytres (CTLs) is associated with rejection of corneal grafts. Transplantation 1995;59:1039-42
13. Kaminski E, Hows J, Goldman J, Batchelor R. Optimising a Limiting Dilution Culture System for quantitating frequencies of alloreactive cytotoxic T lymphocyte precursors. Cellular Immunology 1991;137:88-95
14. Clarke DR, Campbell DN, Hayward AR, Bishop DA. Degeneration of aortic valve allografts in young recipients. J Thorac Cardiovasc Surg 1993;105:934-42
15. Yankah AC, Alexi-Meskhisvilli V, Weng Y, Schorn K, Lange PE, Hetzer R. Accelerated degeneration of allografts in the first two years of life. Ann Thorac Surg 1995;60:S71-77
16. Bouma JB, van der Meer-Prins DM, van Rood JJ, Claas FHJ. Determination of cytotoxic T-lymphocyte precursor frequencies using europium labeling as a nonradioactive to labeling with chromium-51. Human Immunology 1992;35:85-92

17. Koning F, Kordol M, van der Poel J. The influence of workshop monoclonal antibodies on CML, PLT, ADCC, and NK activity. In Reinherz EL., ed. Proceedings of the second international workshop on human leucocyte antigens. Heidelberg: Springer, 1986:189
18. Strijbosch L, Buurman W, Groenewegen G. Limiting dilution assays. Experimental design and statistical analysis. J Immunol Meth 1987;97:133-140
19. O'Brien MF, Stafford EG, Gardner M, Pohlner P, McGiffin D, Johnston N, Brosnan A Duffy P. The viable cryopreserved allograft aortic valve. J Cardiac Surg 1987;1;153-67
20. Mitchell RN, Jonas RA, Schoen FJ. Structural-Functional correlations in cryopreserved allograft cardiac valves. Ann Thorac Surg 1995;60:S108-13
21. Lupinetti FM, Tsai TT, Kneebone JM, Bove EL. Effect of cryopreservation on the presence of endothelial cells on human valve allografts. J Thorac Cardiovasc Surg 1993;106:912-7
22. Mohan R, Feng XJ, Walter P, Herman A. Cryopreserved heart valve allografts can have a normal endothelium. J Thorac Cardiovasc Surg 1994;108:985-7
23. Hoekstra FM, Knoop CJ, Weimar W et al. Stimulation of immune competent cells in vitro by human cardiac valve-derived endothelial cells. Ann Thorac Surg 1995;60:S131-4
24. Strutton G, Hogan P, Green M, O'Brien MF. Immunohistochemical analysis of dendritic cells in human aortic valve allografts. Abstract nr 40 ,World symposium on Heart Valve Disease;1999:p92
25. Salomon RN, Friedman GB, Callow AD, Payne DD and Libby P. Cryopreserved aortic homografts contain viable smooth muscle cells capable of expressing transplantation antigens. J Thorac Cardiovasc Surg 1993;106:1173-80
26. Gould DS, Auchincloss H. Direct and indirect recognition: the role of MHC antigens in graft rejection. Immunology Today 1999;20:77-82
27. Moustapha A, Ross D.B., Bittira B., Lannon C.L., and Lee T.D. Aortic valve grafts in the rat: evidence for rejection. J. Thorac. Cardiovasc. Surg. 1997;114:891-902
28. Green MK, Walsh MD, Dare A, Hogan PG, Zhao XM, Frazer IZ, Bansal AS, O'Brien MF. Histological and immunohistochemical responses after aortic valve allografts in the rat. Ann Thorac Surg 1998;66:S216-20
29. Hoekstra FM, Knoop CJ, Bogers AJ, Weimar W. Donor-specific cellular immune response against human cardiac valve allografts. J Thorac Cardiovasc Surg 1996;112:281-6
30. Tejani AH, Stablein DM, Sullivan EK, Alexander SR, Fine RN, Harmon WE, Kohaut EC. The impact of donor source, recipient age, pre-operative immunotherapy and induction therapy on early and late acute rejections in children: a report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS). Pediatric Transplantation 1998;2:318-24
31. Ettenger RB. Age and the immune response in pediatric renal transplantation. Eur J Pediatr 1992; 151:S7-8
32. Edmunds HL, Clark RE, Cohn LH, Grunkemeier GL, Weisel RD. Guidelines for reporting morbidity and mortality after cardiac valvular operations. J Thorac and Cardiovasc Surg 1996;112:708-11

PART II

EXPERIMENTAL ANIMAL STUDIES

CHAPTER 7

A size-matching heterotopic aortic valve implantation model in the rat

F.B.S. Oei, M.J.P. Welters, F. Bonthuis, L.M.B. Vaessen, R.L. Marquet, P.E. Zondervan, W. Weimar and A.J.J.C. Bogers.

Published in Journal of Surgical Reasearch, 1999, 87; 239-244

Abstract

Structural failure of cardiac valve allografts may be related to technical factors such as size-mismatch, resulting in early intimal proliferation and fibrosis, or immunological reactions against the transplanted valves, featuring lymphocytic infiltration.

To develop a heterotopic aortic valve implantation model in the rat to study the immunological factors leading to graft failure in the setting of a technical adaptation for size-mismatch.

Syngeneic (WAG-WAG or DA-DA) and allogeneic (WAG-BN or WAG-DA) rat strain combinations were used to study the effect of the allogeneic response on the valve properties. An end-to-side anastomosis was made between the U-shaped aortic root graft and the recipient's abdominal aorta to resolve the problems of size matching.

No animals suffered from ischemic or neurological complications during the study period. 100% survival and patency of the aortic grafts was achieved at the end of a 21-day observation period. In the syngeneic group 9 of 10 valves were still competent when assessed during retrograde injection. In contrast, 2 of 10 allogeneic valve grafts were competent at postoperative day 21. Microscopic evaluation revealed no fibrosis or intimal thickening in the syngeneic valve grafts while the allogeneic valve grafts demonstrated rejection-like morphology. The absence of fibrosis and intimal thickening in the syngeneic transplanted valve grafts indicates that this implantation model is not influenced by non-immunological based structural changes. Therefore, this new model enables us to study the association between donor directed immune responses and allograft degeneration in a technically unbiased manner.

Introduction

In heart valve replacement allograft valves are used because of their superior hemodynamic properties [1-3]. As a result, cryopreserved valve allografts gained popularity because of their durability and relatively low incidence of valve related complications compared with other bioprosthetic valves [4,5]. However, aortic and pulmonary valve allografts may fail in the long run. Especially in young children failure of allogeneic heart valves occurs much faster for reasons that are uncertain at the moment [6,7]. A number of studies have demonstrated both humoral and cellular donor-specific immune response after aortic valve implantation in young and adult recipients [8-10]. Recent histological studies on allogeneic heart valves, explanted early or late after implantation, showed signs of both acute and chronic rejection [11,12]. Yet it is still unclear whether, or to what extent, such activation of the immune system is responsible for allogeneic heart valve failure. To investigate the effects of the immune response on valve allografts, *in vivo* studies using an adequate animal model are needed.
Yankah *et al* first described a heterotopic implantation of aortic valve conduits in the rat [13]. Later this model was modified by Green *et al* [14] to solve the anastomotic size-mismatch. In both models the graft is implanted with an interposition technique. To prevent local thrombosis one valvular cusp has to be rendered incompetent to ensure washout of the sinuses of Valsalva (Fig.1).

This alteration of the normal valvular anatomy as well as the mismatch of the anastomotic size may create both surgical trauma and flow turbulence, which could result in accelerated valvular fibrosis and degeneration. Based on the studies of Munger *et al* and v.Kouwenhoven *et al* vascular fibrosis and intimal proliferation is influenced by the surgical technique [15,16]. The proximal anastomotic size-mismatch also excludes transplantation from adult (big) aortic valves into younger (smaller) recipients. This combination is needed to elucidate the reasons for earlier valve failure in young valve recipients. To overcome the drawbacks of the earlier models, Zhou *et al* [17] recently described an end-to-end technique based on a composite graft (Fig.1). This relatively complex model improved size matching between donor and recipient. In addition, it allowed normal mobility of all the three valve cusps. This model also includes practical disadvantages e.g. the need of time consuming supplemental sutures of the valve annulus which could damage the graft.

The aim of this article is to describe a new heterotopic aortic valve implantation technique as a suitable model to study the impact of the immune response on the morphology and function of the aortic allograft without the bias of size mismatch or surgical trauma. In addition, this novel heterotopic implantation model allows the valve to maintain the normal mobility of the three valve cusps and therefore offers the possibility of analyzing the valve competence as a measure for valve function. Meanwhile, by using this model, implantation of adult donor valves into young recipients, in order to study the age factor, seems feasible because of the technical adaptation for the sizemismatch.

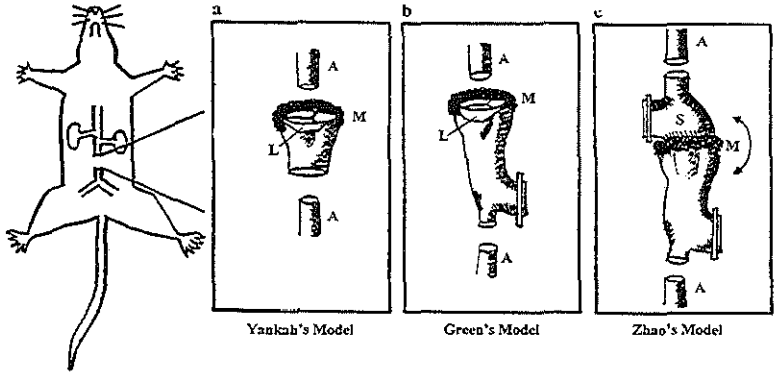


Figure 1:
Heterotopic implantation model by Yankah (Fig.1a) and modified by Green (Fig.1b). Size differences of the aorta annulus and diameter of abdominal aorta can be up to three times. In both models the anterior leaflet is incorporated in the proximal anastomosis to render incompetence of the valve thereby preventing graft thrombosis. The implantation model according to Zhou, using a composite graft, resolves the size-matching problem (Fig 1c). However, this model is time consuming and increases the risk of graft damage. (A) Recipient abdominal aorta. (M) Myocardial rim. (L) Anterior mitral valve leaflet. (S) Additional sutures creating the composite graft.

Materials and Methods

Animals. Male inbred Brown Norway ($RT1^n$/ RyHSD), WAG ($RT1^u$/ RyHSD) and DA ($RT1^a$/RyHSD) rats, ten to twelve weeks old and weighing 200-250 grams, were used in the study (Harlan CPB, Horst, The Netherlands). All animals received food and water *ad libitum*. The experimental protocols were approved by the Committee on Animal Research of the Erasmus University of Rotterdam, the Netherlands, and were concordant with the rules established in the Guidelines on the Protection of Experimental Animals by the Council of the EC (1986).

Donor procedure. Donor rats (WAG or DA) were anaesthetized with ether, followed by an intravenous injection of heparin (100U/100gr). After 3 minutes a median sternotomy was performed followed by *en bloc* removal of both heart and lungs together with the aortic arch and the three arch arteries. By use of an operating microscope at sixfold and tenfold magnification (Zeiss-Ikon, Heidelberg, Germany) the aortic conduit was carefully dissected, in accord with the Standard Operating Procedures of the Dutch Heart Valve Bank Rotterdam. The U-shaped aortic valve conduit consisted of the ascending aorta, aortic arch and descending aorta. At the proximal side of this conduit the ventricular myocardial cuff was minimised, leaving the anterior mitral valve leaflet and approximately 1mm of the myocardial cuff for the proximal anastomosis. The graft was gently flushed with heparinised saline (50U/ml) to clear the lumen. The three aortic valve leaflets were left untouched while both coronary arteries and the three jugular arteries were ligated with 8-0 nylon sutures (Ethicon, Sommerville, NJ). The grafts were kept in cold heparinised saline during preparation of the recipient.

Recipient procedure. After ether anaesthesia, a midline laparotomy was performed. By mobilization of the descending colon the abdominal aorta was exposed. A segment of the abdominal aorta was dissected approximately 1cm below the origin of the renal arteries and approximately 1cm above the aorta bifurcation. This segment was gently cross-clamped with a modified mosquito clamp followed by two incisions within this segment to create the proximal and distal opening for the anastomosis (Fig.2). Both proximal and distal openings were anastomosed with a donor aortic conduit in an end-to-side continues-suturing with 9-0 monofilament nylon sutures (Ethicon). After the clamp was released the native abdominal aorta segment is ligated with 6-0 silk sutures (Ethicon) to complete the bypassing blood flow through the valve conduit. The entire procedure was performed with an operating microscope at 6 to 10 times magnification (Zeiss-Ikon).

Experimental design. To exclude rat strain variability, WAG to BN or WAG to DA allogeneic and WAG to WAG or DA to DA syngeneic transplantations were performed. Five animals were included in each group. After the implantation the patency of the graft was checked twice weekly by palpation of the abdomen.

Based on the results of abdominal aorta transplantation studies, all animals were sacrificed on the 21st post-operative day. After macroscopic examination of the graft, valve competence was analysed by retrograde injection of saline. Subsequently, histological evaluation was performed on all explanted grafts.

Histology. The explanted grafts were longitudinally dissected in three symmetric rectangular pieces. These graft pieces, each containing one valve cusp, were fixed in 10% buffered formalin solution for at least 24 hours. Finally, all graft pieces were embedding in paraffin, cut into 4μm longitudinal sections and stained with standard hematoxylin-eosin.

Analysis: The slides were microscopically examined in a blinded fashion by two independent investigators (FO and MW). Care was taken to avoid analysis of tissue reactions near the sutures. Each slide was scored for the presence of perivascular infiltration of inflammatory cells, retrovalvular thrombosis and preserved leaflets at a 100x magnification. The density of the cellular infiltrate was graded on three categories (none, mild and severe) based on comparison with the normal non-transplanted value.

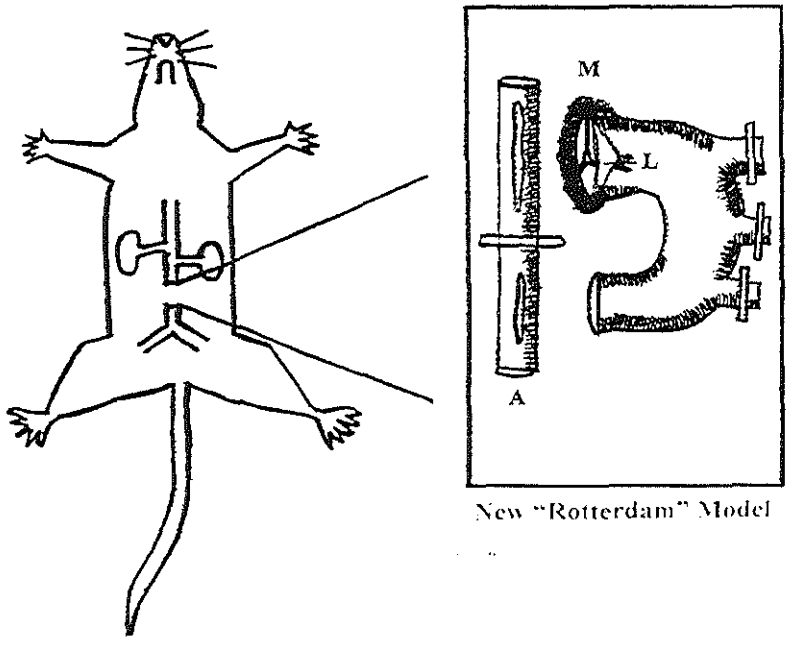


Figure 2:
In the new "Rotterdam" model the normal anatomy of the aortic arch is used to create a U-shaped graft. The jugular arteries are ligated before implantation. After finishing the proximal and distal end-to-side anastomosis, the recipient abdominal aorta will be ligated to ensure the bypass. (A) Recipient abdominal aorta. (M) Myocardial rim. (L) Anterior mitral valve leaflet.

Results

Surgical procedure. The average time needed to complete the donor procedure was 15.3 minutes (range 10 to 20 min.), while the implantation procedure took on average 44.8 minutes (range 35 to 60 min.). No complications occurred during the peri- and postoperative period. All animals recovered without signs of neurological or ischemic disorders of the lower limbs. None of the animals died prematurely during the study. Routine physical examination showed continuous pulsations of the aorta graft in both syngeneic and allogeneic groups indicating that there was still a pulsatile blood flow through an open graft in all the animals until the end of the study period (Table 1a en b). Functional testing of the explanted valve grafts revealed one of 10 valves in the syngeneic group and 8 of 10 valves in the allogeneic group to be incompetent ($p<0.05$)

Table 1 a. Surgical procedures, morphology and competence of the aortic valve graft after 21 days of transplantation

Grafts	Combination	Donor procedure	Implant. time	Open graft	Valve comp.	Preserved leaflets	Perivasc. inflam.	Retrovalv. thrombus
Iso.1	WAG-WAG	15 min	50 min	Yes	Yes	Yes	–	–
Iso.2	WAG-WAG	20 min	40 min	Yes	Yes	Yes	–	–
Iso.3	WAG-WAG	15 min	40 min	Yes	Yes	Yes	+	–
Iso.4	WAG-WAG	10 min	35 min	Yes	Yes	Yes	+	–
Iso.5	WAG-WAG	15 min	40 min	Yes	Yes	Yes	–	–
Iso.6	DA-DA	20 min	50 min	Yes	Yes	Yes	–	–
Iso.7	DA-DA	20 min	45 min	Yes	Yes	Yes	+	–
Iso.8	DA-DA	15 min	40 min	Yes	No	throm.	+	+
Iso.9	DA-DA	15 min	40 min	Yes	Yes	Yes	–	–
Iso.10	DA-DA	15 min	50 min	Yes	Yes	Yes	–	–
Average		**16 min**	**43 min**					

* *Iso. isogeneic; allo. allogeneic; – ,none; +, mild; ++, severe.*

Histology. Macroscopic examination of the valve leaflets showed in 9/10 cases normal morphology in the syngeneic group, whereas leaflets of the allografts appeared shrivelled and dysmorphic. Microscopic analysis of the valves revealed major difference in morphology between the two groups (Table 1a ,b). The syngeneic grafts showed marginal cellular infiltration in the aortic wall, merely in the myocardial rim and the adventitium. Valve leaflets remained vital and cellular in almost all cases, without evidence of thrombosis in the sinuses of Valsalva (9/10) (Fig.3). None of the syngeneic grafts showed proliferation of the intima. The allogeneic grafts were characterised by extensive cellular infiltration in the adventitium and deformed, non-cellular valve leaflets and loss of the extracellular matrix structure (Fig.4). In 5/10 cases, initial formation of retrovalvular thrombi were seen in the Valsalva sinuses. No

thickening or infiltration of inflammatory cells in the tunica intima was seen in the allografts.

Table 1 b. Surgical procedures, morphology and competence of the aortic valve graft after 21 days of transplantation

Grafts	Combination	Donor procedure	Implant time	Open graft	Valve comp.	Preserved leaflets	Perivasc.. inflam.	Retrovalv. thrombus
Allo.1	WAG-BN	15 min	45 min	Yes	No	Deformed	++	–
Allo.2	WAG-BN	20 min	35 min	Yes	No	Thrombus	++	+
Allo.3	WAG-BN	20 min	40 min	Yes	No	Deformed	++	–
Allo.4	WAG-BN	10 min	45 min	Yes	Yes	Deformed	++	–
Allo.5	WAG-BN	15 min	50 min	Yes	No	Thrombus	++	+
Allo.6	WAG-DA	15 min	45 min	Yes	No	Deformed	++	–
Allo.7	WAG-DA	15 min	50 min	Yes	Yes	Deformed	++	–
Allo.8	WAG-DA	10 min	60 min	Yes	No	Thrombus	++	+
Allo.9	WAG-DA	10 min	50 min	Yes	No	Deformed	++	+
Allo.10	WAG-DA	15 min	45 min	Yes	No	Deformed	++	+
Ave.		**16 min**	**43 min**					

Iso, isogeneic; allo, allogeneic; –,none: +, mild; ++, severe.

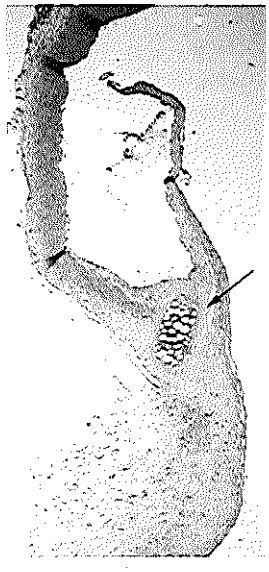


Figure 3:
Longitudinal section of a syngeneic aortic valve graft (WAG to WAG) on postoperative day 21 shows normal valve anatomy with cellular valve leaflets (60-fold magnification, hematoxylin & eosin staining). Marginal lymphocyte-infiltrations are seen in the necrotic myocardium and tunica adventitia. No evidence of thrombosis is seen in the sinus of Valsalva. The cartilage ring of the valve annulus can be used as a anatomical point of reference for the position of valve leaflets (arrow).

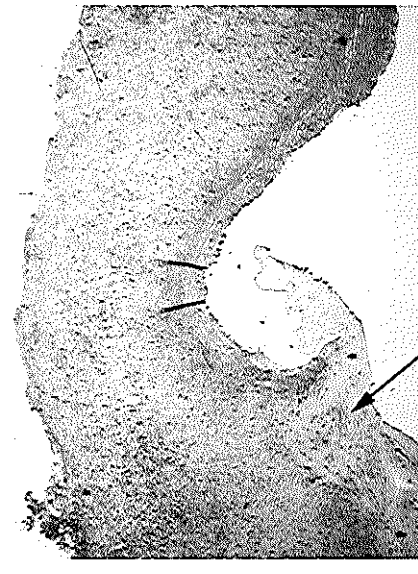


Figure 4:
Longitudinal section of aortic valve allograft (WAG to BN) 21 days after implantation shows deviated valve architecture (60-fold magnification, hematoxylin & eosin staining). The valve leaflet appeared to be shrivelled, edematous and non-cellular. Massive infiltration of mononuclear cells are present in the media and adventitia. The adventitia has been thickened because of the extensive cellular infiltration. Normal anatomy of the valve annulus is seen as a point of reference (arrow).

Discussion

In heart valve transplantation, two crucial questions remain to be answered; i) is the allogeneic valve failure indeed immune-mediated and ii) how does it relate to "age" as a risk factor? To answer these questions an appropriate, technically unbiased *in vivo* experimental model is needed to analyze the pathological changes after transplantation over time in relation to the evoked immune response. In the present study we designed a new, simple heterotopic implantation technique in the rat model, to minimise the influence of non-immunological factors. The existing heterotopic transplantation models in rats are not optimal, because they include several non-immunological factors as causes of graft degeneration. Due to anatomic size differences between the aorta annulus and the diameter of the abdominal aorta the anastomotic size-mismatch may be as great as twofold or even threefold [13,14]. As a result, reactive fibrotic changes in the valve or aneurysms at the anastomosis due to surgical trauma may occur. Our new technique includes a heterotopic transplantation of the aortic valve in the systemic circulation of the rat. The major advantages of this model are the technical adaptation for the size-mismatch of the vascular anastomoses and the maintenance of functional anatomy the three valve cusps. By making end-to-side instead of end-to-end anastomosis, the implantation of larger (older) heart valve grafts into smaller (young) recipients would be possible without excessive surgical manipulation. Compared with other implantation techniques [13,14,17], this new model is less time consuming resulting in a reduction of the operation time. Additionally, this model reduces the ischemic period of both graft and recipient and therefore it could decrease the nonimmunological degeneration and perioperative loss of animals. By maintaining the functional anatomy of the three valve cusps we are able to analyze the valve function, which is measured by the competence of the valve to withstand retrograde injection of saline.

Both syngeneic valves transplants (WAG-WAG and DA-DA) were competent after 21 days and histological examination showed no aberrations. The valve leaflets and vascular wall appeared unaffected and retained their normal cellularity and intercellular matrix. Inflammatory cells were only seen in the myocardial rim and tunica adventitia of the syngeneic valve grafts. This could be the result of a nonspecific inflammatory reaction caused by the concomitantly implanted myocardial rim, which undergoes ischemic necrosis. Kouwenhoven *et al* demonstrated that ischemic time and surgical trauma are immune-independent factors causing intima thickening [16].Histological evaluation of syngeneic valve grafts using earlier techniques revealed unexplained intimal proliferation [11]. Using our implantation model we did not see these intimal thickening in the syngeneic grafts at day 21, which may indicate reduction of the non-immunological factors.

The incompetence of both allogeneic valve grafts (WAG-BN and WAG-DA) at day 21 is most likely due to an immunological process. The observed histological abnormalities, severe peri-vascular infiltration of mononuclear cells, malformation of the leaflet and the presence of thrombi in the sinus Valsalva, strongly suggest such process [11]. Using aortic vascular grafts in the same WAG to BN combination Geerlings *et al* also demonstrated histological aberrations in the vascular wall at 21 days after transplantation, which they

interpreted as histological signs of rejection [18]. Similar histological observations have been published recently for heart valve implants and were also interpreted as signs of rejection [11,19].

From a technical point of view this model has fulfilled our expectations. The simplicity of this implantation procedure will limit the perioperative loss of test-animals and reduce the ischemic time of the graft. It also enables us to study the risk factor "age" by creating a technical adaptation for the size-mismatch. By injecting the allograft in a retrograde direction valve function could be qualified. In addition, there was no histological evidence indicating nonimmunological based structural changes, while the allo-response caused severe damages to the transplanted valves. However, the end-to-side anastomosis used in this model could be a potential disadvantage, because it induces nonlaminar flow into the allograft. Nevertheless, no evidence of any histological changes due to the nonphysiological flow was found at 3 weeks, indicating the absence of hemodynamic influence on valve degeneration shortly after implantation.

Still, the effect of nonlaminar blood flow on long-term valve function has to be evaluated. Another potential drawback of peripheral implantation is the altered valve physiology, which makes this model less suitable for hemodynamic studies of valve allografts. Nevertheless, our implantation technique provides us with a simple, size-matching implantation model, allowing separation of immunological factors from technical factors contributing to structural failure of cardiac valve allografts. Therefore, this model can help us to conduct further research on immunological causes of heart valve allografts deterioration in a more accurate and technically less biased manner.

References

1. Heng M.K., Barratt-Boyes B., Agnew T.M., Brandt P.W., Kerr A.R., and Graham K.J. Isolated mitral replacement with stent-mounted antibiotic-treated aortic allograft valves. *J. Thorac. Cardiovasc. Surg.* 74:230, 1977.
2. Anderson E.T. and Hancock E.W. Long-term follow-up of aortic valve replacement with the fresh aortic homograft. *J. Thorac. Cardiovasc. Surg.* 72:150, 1976.
3. Ross J.K. and Johnson D.C. Mitral valve replacement with homograft, fascia lata and prothetic valve: a long term assessment of valve function. *J. Cardiovasc. Surg.* 15:242, 1974.
4. Barratt-Boyes B.G., Roche A.H., Subramanyan R., Pemberton J.R., and Whitlock R.M. Long-term follow-up of patients with the antibiotic-sterilized aortic homograft valve inserted freehand in the aortic position. *Circulation* 75:768, 1987.
5. O'Brien M.F., McGiffin D.C., and Stafford E.G. Allograft aortic valve replacement: Long-term comparative clinical analysis of the viable cryopreserved and antibiotic 4 degrees C stored valves. *J. Cardiac Surg.* 6:534, 1991.
6. Clarke D.R., Campbell D.N., Hayward A.R., and Bishop D.A. Degeneration of aortic valve allografts in young recipients. *J. Thorac. Cardiovasc. Surg* 105:934, 1993.
7. Kirklin J.K., Smith D., Norvick W., Naftel D.C., and Kirklin J.W. Long-term function of cryopreserved aortic homografts. *J. Thorac. Cardiovasc. Surg.* 106:154, 1993.
8. Hoekstra F.M., Witvliet M., Knoop C.J., Akkersdijk G.P., Bogers A.J., and Weimar W. Donor-specific anti-human leukocyte antigen class I antibodies after implantation of cardiac valve allogarfts. *J. Heart Lung Transplantation* 16:570, 1997.
9. Hogan P., Duplock L., Green M., Smith S., and O'Brien M. Human Aortic valve allografts elicit a donor immune response. *J Thorac. Cardiovasc. Surg.* 112:1260, 1996.
10. Shaddy R.E, Hunter D.D., Osborn K.A., Lambert L.M., McGough E.C., and Fuller T.C. Prospective analysis of HLA immunogenicity of cryopreserved valved allografts used in pediatric heart surgery. *Circulation* 94:1063, 1996.
11. Moustapha A., Ross D.B., Bittira B., Lannon C.L., and Lee T.D. Aortic valve grafts in the rat: evidence for rejection. *J. Thorac. Cardiovasc. Surg.* 114:891, 1997.
12. Rajani B., Mee R.B., and Ratliff N.B. Evidence for rejection of homograft cardiac valves in infants. *J Thorac. Cardiovasc. Surg.* 115:111, 1998.
13. Yankah A.C., Wottge H.U., and Muller-Ruchholtz W. Prognostic importance of viability and a study of a second set allogarft valve: an experimental study. *J. Cardiac Surg.* 3:263, 1988.
14. Green M.K., Zhao X.M., Senewiratne S., and McGiffin D.C. A microsurgical rat model for aortic valve allogarfts. *Transplantation Proc.* 24:2286, 1992.
15. Munger K.A., Coffman T.M., Griffiths R.C., Fogo A., and Badr K.F. The effects of surgery and acute rejection on glomerular hemodynamics in the transplanted rat kidney. *Transplantation* 55:1219, 1993.
16. Kouwenhoven E.A., Marquet R.L., Bonthuis F., IJzermans J.N., and de Bruin R.W. The role of alloantigen-independent factors in transplant arteriosclerosis. *Transplantation Proc.* 29:1721, 1997.
17. Zhou Z.Y., Golshani S.D., Wellisz T., Nimni M.E., and Reinisch J.F. A new model for heterotopic aortic valve transplantation. *Transplantation* 64:228, 1997.
18. Geerlings R.A., de Bruin R.W., Scheringa M., IJzermans J.N., and Marquet R.L. Suppression of acute rejection prevents graft arteriosclerosis after allogeneic aorta transplantation in the rat. *Transplantation* 58:1258, 1994.
19. Green MK, Walsh MD, Hogan PG, Zhao XM, and O'Brien MF Histologic and immunohistochemical responses after aortic valve allografts in the rat. *Ann. Thorac. Surg.* 66:216, 1998.

CHAPTER 8

Heart valve dysfunctioning due to cellular rejection in a novel heterotopic transplantation rat model

F.B.S. Oei, M.J.P. Welters, L.M.B.Vaessen, R.L. Marquet,
P.E. Zondervan, W. Weimar and A.J.J.C. Bogers.

Published in Transplant International, 2000, 13 [Suppl 1]; 528-531

Abstract

Structural failure of heart valve allografts may be related to technical factors or immunological reactions. To circumvent non-immunological factors a new rat implantation model was developed to study whether alloreactivity results in histopathological changes and valve dysfunction.

Syngeneic (WAG-WAG, DA-DA) and allogeneic (WAG-BN, WAG-DA) transplantation was carried out, using this new technique and the function of explanted valves was assessed 21 days later by retrograde comptence testing. Additionally, grafts were examined using standard histological and immunohistochemical techniques.

There was no leakage during retrograde injection in 9/10 syngeneic and 2/10 allogeneic grafts. Microscopically, syngeneic valves appeared normal without fibrosis or intimal thickening, although $CD8^+$ lymphocytes and macrophages were found in necrotic myocardial rim and adventitia. In contrast, allogeneic valves were deformed and noncellular with extensive infiltration of $CD4^+$, $CD8^+$ and $CD68^+$ cells in adventitia and media.

Absence of fibrosis and intimal thickening in syngeneic transplanted valves indicated circumvention of nonimmunological factors. Allogeneic valve transplantation induces cellular infiltration in the graft with subsequent graft failure.

Introduction

Human aortic valve allografts (AVA), as used in valve replacement surgery, were successfully introduced in the 1960's [11]. To improve clinical performance and durability of these AVAs different methods of decontamination and storage have been evaluated [1]. The rationale is to preserve viable fibroblasts in these valves. Fibroblasts are needed for maintenance of the extracellular matrix in the valve, thereby preventing structural valve deterioration. O'Brien *et al.* introduced cryopreservation as a method for viable storage of heart valves and experienced good clinical results [9]. On the other hand, implantation of human heart valves with viable cells could evoke a specific immune response, which could be the reason for early graft failure, which often is observed in children [2].

Several experimental and clinical studies have demonstrated the immunogeneicity of AVAs, even after cryopreservation [4,13]. Yet, it is still unclear whether, or to what extent, activation of the immune system by AVA implantation is responsible for valve allograft failure. To investigate the relationship between the immune response and the degeneration of AVAs, an adequate *in vivo* model is required.

Current *in vivo* transplantation models in the rat include heterotopical interposition of the AVA in the abdominal aorta [3,12]. To prevent local thrombosis one valvular cusp has to be rendered incompetent so that washout of the sinuses of Valsalva is ensured. The adjustment of the valvular anatomy and the mismatched anastomosis, owing the circumferential difference between the aortic annulus and the abdominal aorta, may create both surgical trauma and flow turbulence. As a result accelerated valvular fibrosis and intimal proliferation may occur [6]. Therefore, the current AVA transplantation models are not suitable for evaluating the effect of the alloreactivity on the morphology and function of the AVA.

The aim of the present study is to evaluate a new heterotopic aortic valve implantation technique as a suitable model to study transplantation of AVAs without the bias of size-mismatch or surgical trauma. This new implantation technique allows the valve to maintain the normal mobility of the three valve cusps and therefore includes the possibility to analyse the valve competence as a measure for valve function. With this model we were able to investigate the histological and functional consequences of the immune response to the MHC-mismatched donor AVA, without technical bias.

Material and Methods

Animals. Male inbred Brown Norway (RT1^n/ RyHSD), DA (RT1^a/RyHSD) and WAG (RT1^u/ RyHSD) rats (Harlan CPB, Horst, The Netherlands), weighing 200-250 grams, were used. All animals received food and water *ad libitum*. The experimental protocols were approved by the Committee on Animal Research of the Erasmus University of Rotterdam, the Netherlands, and were concordant with the Principles of Laboratory Animal Care (1985).

Donor procedure. The heart of the anaesthetised donor rats (WAG or DA) was removed, followed by dissection of the U-shaped aortic valve conduit. The graft, consisting of the myocardial rim, ascending aorta, aortic arch and descending aorta, was flushed with heparinised saline (50U/ml). After ligation of the coronary and jugular arteries with 8-0 nylon sutures (Ethicon, Sommerville, NJ), the grafts were stored in cold heparinised saline until transplantation.

Recipient procedure. After ether anaesthesia, midline laparotomy was performed. The infra-renal part of the abdominal aorta was dissected and cross-clamped with a modified mosquito clamp. Two incisions were made for the end-to-side anastomoses using continuous 9-0 monofilament nylon sutures (Ethicon) (see Figure 2, Chapter 7). The native abdominal aorta segment was ligated with 6-0 silk sutures (Ethicon) to complete the bypassing blood flow through the graft. *Study design.* To exclude rat strain variability, WAG to BN or WAG to DA allogeneic and WAG to WAG or DA to DA syngeneic transplantations were performed. There were five animals in each group. After implantation, the patency of the graft was checked twice weekly by palpation of the abdomen. All rats were sacrificed on post-operative day 21 and the AVA removed. Qualitative functional examination of the graft was performed by retrograde injection of saline. Subsequently, the grafts were dissected longitudinally in three symmetric pieces, each containing one valve cusp, and prepared for histological and immunohistochemical evaluation.

Histology. After fixation in 10% buffered formalin solution for at least 24 hours and embedding in paraffin, longitudinal sections of 4 μm were cut, followed by staining with standard hematoxylin-eosin.

Immunohistochemistry. After embedding in Tissue-Tek (Miles Diagnostic Division, Elkhart, IN) and frozen in liquid nitrogen, 5 μm cryosections were stained by the three-layer immunoperoxidase technique as described previously [5]. The primairy antibodies (Serotec, Oxford, UK) used were: W3/25 (CD4; dilution 1:300), ED1 (CD68; dilution 1:200), Ox8 (CD8; dilution 1: 300) and an irrelevant IgG as a negative control.

Analysis. The slides were microscopically examined (blinded) by two independent investigators. Each slide was scored for the presence of preserved valve leaflets, perivascular infiltration, retrovalvular thrombosis and inflammatory cells. For comparison, normal non-transplanted valves were evaluated.

Results

Transplantation procedure. The time needed to obtain and prepare the donor valve was comparable for all rats and ranged between 10 to 20 minutes, while the implantation procedure time varied from 35 to 60 minutes (Table 1a). Routine physical examination showed continuous pulsations of the AVA in all the animals until the end of the study period (postoperative day 21), but functional testing of the explanted valves revealed 1/10 of the syngeneic and 8/10 of the allogeneic group to be incompetent (Table 1a; student t-test $p < 0.05$).

Table 1a. Surgical procedures, morphology and competence of the aortic valve graft after 21 days of transplantation

Graft[1]	Combination	Donor proc. min[2].	Impl. time min[3]	Valve comp[4]	Preserved leaflets	Perivasc. inflam[5]	Throm[6]	CD 4+ cells[7]	CD 8+ cells[8]	CD 68+ cells[9]
Syn.1	WAG-WAG	15 m	50 m	Yes	Yes	–/++	–	–	–	–
Syn.2	WAG-WAG	20 m	40 m	Yes	Yes	–	–	nd[11]	nd	nd
Syn.3	WAG-WAG	15 m	40 m	Yes	Yes	+[10]	–	–	A[12]	A
Syn.4	WAG-WAG	10 m	35 m	Yes	Yes	+	–	–	A	A
Syn.5	WAG-WAG	15 m	40 m	Yes	Yes	–	–	nd	nd	nd
Syn.6	DA-DA	20 m	50 m	Yes	Yes	–	–	nd	nd	nd
Syn.7	DA-DA	20 m	45 m	Yes	Yes	+	–	nd	nd	nd
Syn.8	DA-DA	15 m	40 m	No	Thromb[6]	+	+	nd	nd	nd
Syn.9	DA-DA	15 m	40 m	Yes	Yes	–	–	nd	nd	nd
Syn.10	DA-DA	15 m	50 m	Yes	Yes	–	–	nd	nd	nd
Ave		**16 m**	**43 m**							

[1]Syn, syngeneic; Allo, allogeneic; [2]Donor proced., donor procedure time; [3]Implant. time, heart valve implantation time; [4]Valve comp., Valve competence; [5]Perivasc. inflam., Perivascular inflammation; [6]Thrombus, Retrovascular thrombus; [7]CD4+ cells, cells stained positively with mouse-anti-rat antibody W3/25; [8]CD8+ cells, cells stained positively with mouse-anti-rat antibody Ox8; [9]CD68+ cells, cells stained positively with mouse-anti-rat antibody ED1; [10] -, none; +, mild; + +, severe; [11] nd, not determined; [12]A, adventitia; I, intima; M, media;.

Explanted grafts. Macroscopically, 9/10 syngeneic valve leaflets showed normal morphology, whereas all allogeneic leaflets were shrivelled and dysmorphic (Table 1a,b; preserved valve leaflets). Microscopic analysis showed major structural difference between the two groups (Table 1a,b). The valve leaflets in syngeneic grafts appeared normal and retrovalvular thrombosis occurred only in one rat. No early fibrosis or intima proliferation was observed. Marginal cellular infiltrates were found in the myocardial rim and the adventitium of these syngeneic grafts (Table 1a,b; perivascular inflammation). Immunohistochemical evaluation of 3 syngeneic AVAs showed that the cellular infiltration consisted mainly of macrophages ($CD68^+$) and some cytotoxic T cells ($CD8^+$) (Table 1a,b). The allogeneic valve leaflets were deformed and non-cellular as well as loss of extracellular matrix structure.

Table 1 b. Surgical procedures, morphology and competence of the aortic valve graft after 21 days of transplantation

Grafts[1]	Combination	Donor Proc. min[2]	Implant. time min[3]	Valve comp[4]	Preserved leaflets	Perivasc. inflam[5]	Thromb[6]	CD 4+ cells[7]	CD 8+ cells[8]	CD 68+ cells[9]
Allo.1	WAG-BN	15 m	45 m	No	Deformed	++[10]	−[10]	I/A[12]	I/M/A[12]	I/M/A
Allo.2	WAG-BN	20 m	40 m	No	Thrombus[6]	++	+[10]	A	M/A	M/A
Allo.3	WAG-BN	10 m	45 m	Yes	Deformed	++	−	A	A	M/A
Allo.4	WAG-BN	15 m	50 m	No	Thrombus	++	+	nd[11]	nd	nd
Allo.5	WAG-BN[13]	20 m	50 m	No	Deformed	++	−	A	A	M/A
Allo.6	WAG-DA	15 m	45 m	No	Deformed	++	−	nd	nd	nd
Allo.7	WAG-DA	15 m	50 m	Yes	Deformed	++	−	nd	nd	nd
Allo.8	WAG-DA	10 m	60 m	No	Thrombus	++	+	nd	nd	nd
Allo.9	WAG-DA	10 m	50 m	No	Deformed	++	+	nd	nd	nd
Allo.10	WAG-DA	15 m	45 m	No	Deformed	++	+	nd	nd	nd
Ave		**15 m**	**47 m**							

See legend of Table 1a. [13]Recipient rat sacrificed 51 days instead of after 21 days after allogeneic heart valve transplantation.

Furthermore, in 6/10 cases, initial formation of retrovalvular thrombi were seen in the Valsalva sinuses. No thickening or infiltration of the tunica intima were seen, except in one explanted graft. In the allogeneic grafts studied immunohistochemically, the adventitia was extensively infiltrated with $CD4^+$, $CD8^+$ and $CD68^+$ cells (Table 1a,b). Additionally, in these grafts the amount of elastin fibers in the media was reduced and $CD68^+$ cells were found between these fibers.

Discussion

The effect of the immune response on the morphological and functional changes of AVAs is still unclear and can only be studied using a suitable *in vivo* model. Previous heterotopic transplantation models in rats are not appropriate, because several nonimmunological factors mask the immune-specific morphological changes. In the present study, a new model for heterotopic implantation of AVAs in rats was used to determine structural and functional consequences of the antidonor immune response on the AVA transplanted across a MHC barrier. A technical adaptation of the anastomotic mismatch (end-to-side anastomosis) made it possible to reduce the implantation time and surgical trauma. Consequently, no peri- and post-operative complications were observed. In addition, by adapting the size-mismatch, the non-immunological degeneration, characterized by early fibrosis and intimal proliferation in the syngeneic transplantation [6], was minimized. Syngeneic transplanted AVAs, using the "Rotterdam" model, showed no histological evidence of structural changes, while allogeneic transplanted AVAs were severely damaged. Therefore, this novel model can be used to study the morphological and functional consequences of an allogeneic immune response. Our data indicate that the vascular wall of fresh AVAs implanted across a MHC barrier were infiltrated by mononuclear cells (MNC), while the valve leaflets were deformed and non-cellular. These infiltrating T-lymphocytes ($CD4^+$ and $CD8^+$) and macrophages ($CD68^+$) are regarded as effector cells in solid organ and tissue rejection. Their appearance is localized mainly in the tunica adventitia and the outer layer of the tunica media of the aortic wall and not in the intimal layer, except for one explanted graft. This phenomenon could be explained by the abundancy of endothelial cells in the complex vascular network of the adventitia, while the tunica intima only bares a monolayer of endothelial cells. These endothelial cells are regarded as the main target cells in the immune response directed against AVA [7]. The noncellularity and deformation of the valve leaflets at 3 weeks after transplantation is probably the result of early cellular destruction of the leaflet components.

MNC were also observed at 21 days after transplantation in the syngeneic aortic vascular wall. This infiltration is totally different from the allogeneic transplanted valve in cell type, intensity and localization. Only $CD68^+$ cells (macrophages) were present predominantly in the necrotic myocardial rim and a minority of these cells was found in the adjacent adventitium of the aortic wall. The necrotic tissue probably induces this nonspecific immune response and subsequently the spread of cellular infiltration into the adventitia [8].The histological signs of cellular infiltration and graft injury corresponded to the results of functional analysis of the AVAs; that is to say extensively infiltrated valves showed more leakage. This study demonstrates that AVAs induce invasive and destructive cellular immune responses when implanted in a recipient with MHC disparity. In addition, cellular infiltration and destruction do indeed result in valve dysfunction. Still

further studies using the "Rotterdam" implantation model are needed to evaluate the kinetics of the cellular immune response and valve dysfunction. Further relevant issues include the role of cryopreservation and immunosuppression in modifying valve infiltration and injury are currently under investigation.

References

1. Barratt-Boyes BG . A method for preparing and inserting a homograft aortic valve. Br J Surg 1965; 52: 847.
2. Baskett RJ, Ross DB, Nanton MA, Murphy DA. Factors in the early failure of cryopreserved homograft pulmonary valves in children: preserved immunogenicity ? J Thorac Cardiovasc Surg, 1996;112: 1170-79.
3. Green MK, Zhao XM, Senewiratne S, McGiffin DC. A micro-surgical rat model for aortic valve allografts. Transplantation Proc 1992; 24: 2286. 1962
4. Hoekstra FM, Witvliet M, Knoop CJ, Bogers AJ, Weimar W. Donor-specific anti-human leukocyte antigen class I antibodies after implantation of cardiac valve allogarfts. J Heart Lung Transplantation 1997;16: 570-72.
5. Kouwenhoven EA, de Bruin RWF, Heemann UW, Marquet RL, IJzermans JNM. Late graft dysfunction after prolonged cold ischemia of the donor kidney. Inhibition by cyclosporin. Transplantation 1999.
6. Kouwenhoven EA, Marquet RL, Bonthuis F, IJzermans JN, de Bruin RW. The role of alloantigen independent factors in transplant arteriosclerosis. Transplantation Proc 1997;29: 1721-22.
7. Lupinetti FM, Christy JP, King DM, El Khatib H, Thompson SA. Immunogenicity, antigeneicity and endothelial viability of aortic valves preserved at degrees C in a nutrient medium. J Cardiac Surg 1991; 6: 454-61.
8. Moustapha A, Ross DB, Bittira B, Lannon CL, Lee TD. Aortic valve grafts in the rat: evidence for rejection. J Thorac Cardiovasc Surg 1997; 114: 891-902.
9. 'O Brien MF, McGiffin DC, Stafford EG. Allograft aortic valve replacement: Long-term comparative clinical analysis of tyhe viable cryopreserved and antibiotic 4 degree C stored valves. J Cardiac Surg 1991; 6: 534-535.
10. Pacifico AD, Kirklin JW. Homografts for replacement of the aortic valve. Circulation 1977; 55: 353-61.
11. Ross DN. Homograft replacement of the aortic valve. Lancet 1962; 2: 487-90.
12. Yankah AC, Wottge HU, Muller-Ruchholtz W. Prognostic importance of viability and a study of a second set allogarft valve: an experimental study. J Cardiac Surg 1988; 3: 263-64.
13. Zhao XM, Green M, Frazer IF, Hogan P, O'Brien MF. Donor-specific immune response after aortic valve allografting in the rat. Ann Thorac Surg 1994; 57: 1158-63.

CHAPTER 9

Frequencies of donor reactive helper T lymphocytes correlate with rejection of fresh allogeneic aortic valve grafts in rats

F.J.M.F. Dor, F.B.S. Oei, L.M.B. Vaessen, R.L. Marquet, A.J.J.C. Bogers and W. Weimar.

Submitted for publication in the Journal of Heart Valve Disease
Published in a revised form in Transplantation Proceedings, 2001;33:640-641

Abstract

Human valve allografts are commonly used in cardiac surgery for congenital and acquired valve diseases. Particularly in the pediatric population, these allografts are prone to fail on the long-run and require replacement. In part this failure is regarded to be due to immunological phenomena. In this regard, the frequency of helper T lymphocytes (HTL) measured in peripheral blood and spleen is a parameter for acute rejection in organ transplantation. The value of this parameter in fresh heterotopic valve transplantation was studied using the 'Rotterdam' implantatation model in rats.
Frequencies of donor-reactive IL-2 producing HTL were determined in peripheral blood and spleen at 7 and 21 days after allogeneic (WAG→DA) and syngeneic (DA→DA) implantation of a fresh aortic valved conduit. Pre-implantation and after explantation at day 7 and 21, valve competence was tested by a retrograde saline injection. Histological examination of explanted valves was performed after haematoxilin-eosin staining of the parafin sections. Allogeneic (WAG) skin graft recipients served as positive controls.
Seven days after transplantation, the donor-reactive HTL frequencies (HTLf) in the spleen (median:71/10^6) but not in peripheral blood (median:26/10^6) of rats receiving an allogeneic graft were significantly elevated compared to frequencies of pretransplant and syngeneic transplanted rats. In the allogeneic group at day 21 after transplantation, a significant increase in HTLf was seen in both peripheral blood (median: 109/10^6) and spleen (median: 92/10^6) compared to pretransplantation and the syngeneic groups. All 5 (100%) syngeneic grafts and 5/7 (71%) allografts were competent at day 7. At day 21, 5/5 syngeneic grafts (100%) and 0/5 allografts (0%) remained competent (p=0.01). Histologically, mononuclear cell infiltration in the allogeneic valve leaflets and in the vascular wall was observed at day 7. At day 21 valve leaflets appeared to be acellular and deformed. All syngeneic valve grafts retained normal morphology. *Conclusions:* After allogeneic aortic valve transplantation in the rat, the frequency of donor-reactive HTL correlates with valve dysfunction and histopathological signs of rejection, suggesting the role of immune reactivity in valve allograft destruction. Therefore, the analysis of donor-directed HTL frequencies may be a useful tool in monitoring the cellular immune response as an indicator for early graft dysfunction as a result of rejection in clinical valve transplantation.

Introduction

Human cardiac valve allografts are widely applied for the treatment of acquired and congenital cardiac diseases. Aortic or pulmonary valve allografts have excellent hemodynamic properties and a relatively low incidence of valve related complications in comparison to other bioprostheses (1,2). Despite their advantages, long-term results in clinical valve transplantation are not yet optimal (3,4). Especially in children valve failure occurs more early for reasons that are not completely understood (5,6). The theory of immune-related valve deterioration is based on the fact that valve allograft transplantation is performed without Human Leucocyte Antigen (HLA) matching between donor and recipient. Furthermore, valve allograft recipients are not immune-suppressed. Theoretically, implantation of allogeneic material under these circumstances may ultimately lead to rejection of the transplanted tissue. Several studies have demonstrated the capacity of valve allografts to evoke a donor-specific humoral immune response in vivo (6,7). Nevertheless, the correlation of this donor-specific immune activation and structural valve deterioration remains unclear.

In the clinical setting monitoring the function of valves allografts after transplantation as relevant evidence of tissue rejection is very difficult. Echocardiographical follow-up of the valve allograft can be used for evaluation of the valve function, but it's value for the diagnosis of graft rejection is questionable. Unlike in organ transplant recipients, no biopsies are available for monitoring the tissue injury e.g. cellular graft infiltration or tissue necrosis. The only possibility to study the graft histologically is at time of reoperation or autopsy, considered to be the end-stage of valve failure. At this point, immunological reactions may already be extinguished.

Helper T lymphocytes (HTL) directed against alloantigens play an important role in the immunological effector mechanisms that underlie allograft rejection (8). After solid organ transplantation, frequencies of HTL have been correlated with transplantation outcome. An increase in the number of circulating donor reactive HTL in peripheral blood was measured at the time of histologically diagnosed rejection (9,10). During periods of immunological quiescence, the frequency of donor reactive HTL stayed at baseline (10). The aim of the present study is to investigate the relevance of donor reactive HTL frequencies in peripheral blood and the spleen of fresh aortic valve allografts recipients in respect to the function and histo-morphology of the allograft.

Materials and Methods

Animals. Ten to twelve weeks old (200-275 grams) inbred male DA rats (RT-1^a /OlaHSD, Harlan, Horst, The Netherlands) were used as recipients and syngeneic donors. Inbred male WAG rats (RT-1^u/RyHSD, Harlan) of comparable age were used as allogeneic donors. All animals received food and water ad libitum. The experiments were in compliance with the Guidelines on the Protection of Experimental Animals by the Council of the EC (1986) and were approved by the Committee on Animal Research of the Erasmus University Rotterdam, The Netherlands.

Experimental design. Surgical procedures were performed according to the "Rotterdam" heterotopical implantation model (11). Twelve DA rats received an allogeneic (WAG) graft, and ten DA rats a syngeneic (DA) valve graft. Rat strain variability has been excluded in earlier experiments (11). Prior to transplantation, 1 ml of peripheral blood of each rat was collected from the tail vein to determine the pretransplantation HTLf. Five animals of the syngeneic and 7 of the allogeneic group were sacrificed 7 days after transplantation, whereas 5 allograft recipients and 5 rats of the syngeneic group were sacrificed on post-operative day 21. Peripheral blood was collected and splenectomy performed for cellular testing (HTLf determination). Before implantation and after explantation, a qualitative functional examination of the graft was performed by retrograde injection of saline. The grafts were dissected longitudinally in three symmetric rectangular pieces, each containing one valve cusp and were fixed in 10% buffered formaline solution for at least 24 hours and embedded in paraffin. Thereafter, longitudinal sections of 4 μm were cut, followed by standard staining with hematoxylin-eosin. Two independent investigators examined the slides microscopically in a blinded fashion.
As positive control experiment for the HTLf analysis, three naive DA rats received an allogeneic (WAG) full thickness skin graft (1.5 cm^2). The grafts were sutured onto comparable defects created in the recipients' neck. The skin grafts were protected by a soft wire gauze for daily dressing after inspection. Peripheral blood was collected from the tail vein before and every 7 days after skin grafting. Twenty-one days postoperatively, the rats were splenectomized and sacrificed.

Isolation of PBMC. One ml of the collected heparinized blood was transferred into a 10-ml tube containing 4 ml of Hanks' Balanced Salt Solution (HBSS, BioWhittaker, Verviers, Belgium) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (BioWhittaker). PBMC were isolated by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) during 20 minutes at 900g with no brake. PBMC were harvested from the interphase and washed two times in excess with HBSS by centrifugation at 400g for 5 minutes. After these washing steps, the cell pellet was suspended into 1 ml of Rat Culture Medium (RCM, RPMI 1640 (BioWhittaker) supplemented with 100 IU/ml Penicillin and 100 μg/ml Streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker), 10 mM Hepes (Biochrom KG, Berlin,

Germany), $2x10^{-5}$ M β-mercapto-ethanol (Serva Feinbiochemica, Heidelberg, Germany)) and 10% 30' heat-inactivated (56°C) Fetal Calf Serum (Hyclone, Logan, Utah, USA).
The number of PBMC was determined by using a Bürker cell count chamber. PBMC were tested for their viability by stimulation during 72h with and without 5 μg/ml Concanavalin A (Con A, Amersham Pharmacia Biotech, Uppsala, Sweden) in triplicate. The last 8 hours of incubation, the cultures were labeled with 0.5 μCi/well ^{3}H-TdR (Amersham Pharmacia Biotech). Mean counts per minute (cpm) were determined and expressed as stimulation index (SI) by dividing the cpm obtained in Con A presence by the cpm without Con A.

Preparation of spleen cells. Spleen cells were obtained by mechanical dissociation of small pieces of spleen through a sieve of stainless steel in HBSS. Subsequently the cell suspension was filtrated through a 70-μm cell strainer (type 2350 Becton Dickinson Labware, New Jersey, USA). Thereafter, the cells were centrifuged over a Ficoll-Paque density gradient (Pharmacia Biotech AB, Uppsala, Sweden). The cells from the interphase were collected and washed two times with HBSS. After the washing steps, the cell pellet was suspended into 1 ml of RCM supplemented with 10% 30' heat-inactivated (56°C) Fetal Calf Serum (Hyclone, Logan, Utah, USA). Cell counts were obtained by using a Bürker cell count system. Fresh responder spleen cells were used in the limiting dilution assay for HTLf determination. Fresh stimulator spleen cells (WAG for allogeneic stimulation) were irradiated (30 Gy) and added to the responder cells in the LDA. Viability of the spleen cells was tested with Con A stimulation as described above for fresh PBMC.

Limiting Dilution Assay for HTLf determination. Twelve replicates of graded numbers of fresh responder (DA) PBMC were titrated in seven-fold double dilutions starting from $1,0x10^5$ cells/well down to 1563 cells/well in U-bottomed microtiter plates (Costar, Cambridge, MA). Responder spleen cells were titrated in seven-fold double dilutions starting from $0,4x10^5$ cells/well down to 625 cells/well. As stimulator cells $5x10^4$ 30 Gy irradiated WAG spleen cells were used for allogeneic stimulation. The cells were cultured in a total volume of 200 μl RCM supplemented with 10% heat-inactivated (30' 56° C) Fetal Calf Serum (FCS, Hyclone). After 72 hours of incubation at 37°C in a humidified atmosphere containing 5% CO_2, 100 μl of supernatant of each well were transferred to U-bottom trays and frozen at -20°C for later HTL frequency determination in a bioassay (see below)

IL-2 bioassay. The murine cytotoxic T lymphoblastic line CTLL-2, sensitive to IL-2 but not to IL-4 (12) was cultured in 25 cm^2 flasks (Costar). The culture medium existing of RPMI-1640 (BioWhittaker), 10% of 30' heat-inactivated (56°C) Bovine Calf Serum (BCS, HyClone), $2x10^{-5}$ M β-mercapto-ethanol (Serva Feinbiochemica) and 10 IU/ml IL-2 Lymphocult T (Biotest AG, Dreieich, Germany). Every two days the culture medium was renewed. The culture medium was renewed for the last time 48 hours prior to use as an indicator for IL-2 production in the LDA cultures. Shortly

before use the CTLL-2 cells were washed 3 times and maintained in test medium (culture medium without IL-2) at a concentration of $5x10^4$ / ml. On the day of IL-2 determination the U-bottom trays containing the 100 µl of supernatant of the LDA were thawed at 37°C in a humidified atmosphere containing 5% CO_2. After thawing, 100 µl of the indicator cell suspension containing $5x10^3$ CTLL-2 were added to each well. After 20 hours of incubation at 37°C in a humidified atmosphere with 5% CO_2, 20 µl (0,5 µCi) ^{3}H-TdR (Amersham Pharmacia Biotech) were added to each well. After another 4 hours of incubation, the plates were harvested using a Skatron Basic 96 Harvester (Skatron Instruments, Lierse, Norway). Proliferation of the CTLL-2 cells was determined by ^{3}H-TdR incorporation measured using a Wallac 1205 BetaPlate scintillation counter (LKB-Wallac Instruments, Turku, Finland).
Frequencies of (donor reactive) HTL were calculated as described by Strijbosch et al (13) . The proportion of negative wells at each concentration of responder cells is linearly related to the frequency of responder cells, according to the Poisson distribution: $-\log_e P_{neg} = fX$, where P_{neg} is the proportion of negative wells, f is the frequency of responder wells and X is the sample size of responder cells per well. Cultures were considered to conform to single hit kinetics, i.e. one single cell of one cell type is responsible for the observed IL-2 production in a culture well, when the goodness of fit was less than 12.5 calculated by Jackknife analysis. Individual frequencies were regarded as different if their 95% confidence intervals were not overlapping.

Statistical analysis. The Fisher's Exact Test, the Mann-Whitney Test and the paired T-test were used when applicable, and performed with GraphPad Prism (GraphPad Software Inc., San Diego, USA).

Results

Transplantation procedure. Results of the surgical procedures are listed in table 1and 2. All transplantated animals recovered well and did not suffer from post-operative neurological or ischaemic problems. Palpable pulsations in the abdomen were checked on regular basis and all valve grafts were open until the end of the study.

Table 1. Surgery, histology and valve competence 7 days after transplantation

Grafts	Combination	Donor proced.	Implant. Time	Open graft	Valve comp.	Preserved valve leaflets	Perivasc. Inflamm.	Thrombus
S 7.1	DA-DA	15	40	Yes	Yes	Yes	-	-
S 7.2	DA-DA	10	50	Yes	Yes	Yes	-	-
S 7.3	DA-DA	15	45	Yes	Yes	Yes	-	-
S 7.4	DA-DA	15	45	Yes	Yes	Yes	-	-
S 7.5	DA-DA	15	25	Yes	Yes	Yes	-	-
A 7.1	WAG-DA	25	45	Yes	Yes	Deformed	+	+
A 7.2	WAG-DA	15	35	Yes	Yes	Yes	+	-
A 7.3	WAG-DA	15	45	Yes	No	Deformed	+	+
A 7.4	WAG-DA	15	60	Yes	Yes	Yes	+	-
A 7.5	WAG-DA	20	50	Yes	Yes	Yes	+	-
A 7.6	WAG-DA	15	35	Yes	No	Yes	+	+
A 7.7	WAG-DA	20	45	Yes	Yes	Yes	+	-

S, syngeneic; A, allogeneic; 7.x, rat number x seven days after transplantation; Duration of surgical procedures are given in minutes; -, none; +, mild; ++, severe.

Valve competence and histology. Functional testing of the explanted valves revealed no significant difference in incompetence between syngeneic (0/5) and allogeneic (2/7) valve transplants at postoperative day 7. Twenty-one days after transplantation 0/5 of the FS and 5/5 of the FA were not competent (p=0.01).

Microscopic evaluation revealed normal valve morphology in syngeneic transplanted rats at seven days postoperatively (Fig 1). In the allogeneic group, in 5/5 moderate perivascular infiltration was seen, in 2/5 the valve leaflets were deformed and in 2/5 retrovalvular thrombosis was present (Table 1) (Fig 2). Twenty-one days after syngeneic transplantation, no histological pathology could be found (Fig 1). In allogeneic transplanted rats, severe perivascular infiltration was seen in 5/5 animals and all valve leaflets appeared acellular and dysmorphic (Fig. 2). In 3/5 rats, retrovalvular thrombosis was present (Table 2).

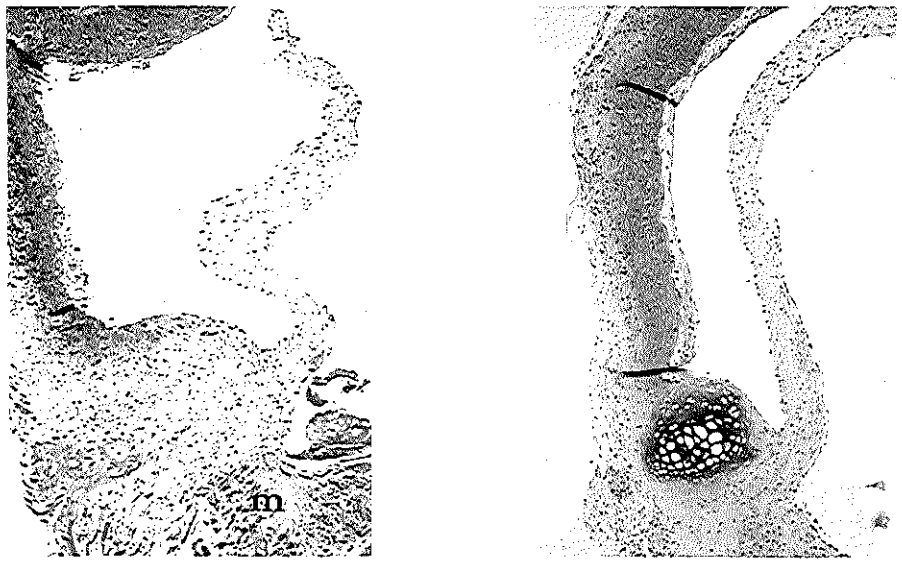


Figure 1. Longitudinal section of a syngeneic aortic valve graft (DA to DA) on postoperative day 7 (left) and 21 (right). Both slides demonstrate normal valve anatomy and cellular valve leaflets (60-fold magnification, hematoxylin & eosin staining). Only minor mononuclear cell infiltration is seen in the necrotic myocardial rim and tunica adventitia (m).

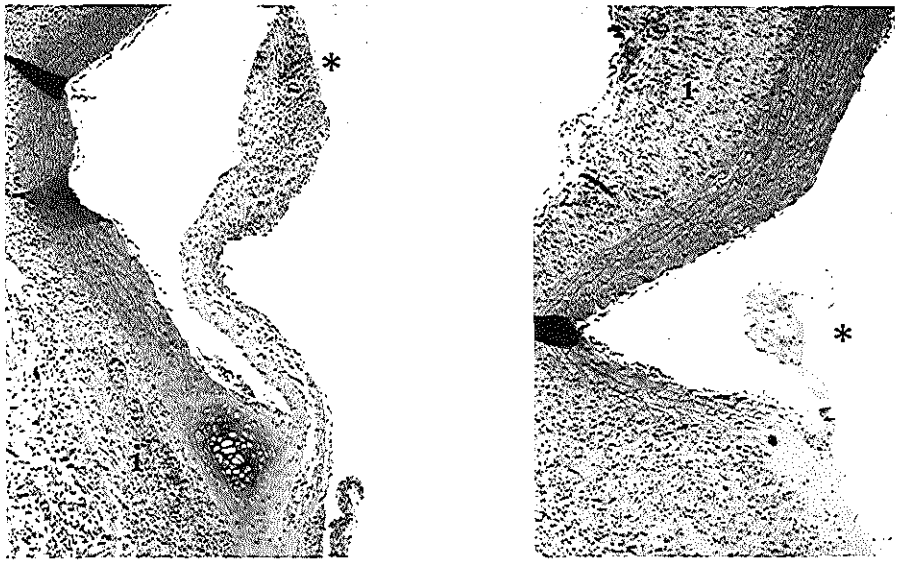


Figure. 2. Longitudinal section of an allogeneic aortic valve graft (WAG to DA) on postoperative day 7 (left) and 21 (right) (60-fold magnification, hematoxylin & eosin staining). Note the massive lymphocyte infiltration in aortic wall (i) and valve leaflet (*) already present at day 7 (left). On day 21 the valve leaflet appeared to be shrivelled and non-cellular (*), with extensive mononuclear cell infiltrations in the adventitia (i) (right).

Table 2. Surgery, histology and valve competence 21 days after transplantation

Grafts	Combination	Donor proced.	Implant. Time	Open graft	Valve comp.	Preserved valve leaflets	Perivasc. Inflam.	Thrombus
S 21.1	DA-DA	20	50	Yes	Yes	Yes	–	–
S 21.2	DA-DA	20	45	Yes	Yes	Yes	–	–
S 21.3	DA-DA	15	40	Yes	Yes	Yes	–	–
S 21.4	DA-DA	15	40	Yes	Yes	Yes	–	–
S 21.5	DA-DA	30	35	Yes	Yes	Yes	–	
A 21.1	WAG-DA	15	45	Yes	No	Deformed	++	+
A 21.2	WAG-DA	15	50	Yes	Yes	Deformed	++	–
A 21.3	WAG-DA	15	45	Yes	No	Deformed	++	+
A 21.4	WAG-DA	15	30	Yes	No	Deformed	++	+
A 21.5	WAG-DA	15	35	Yes	No	Deformed	++	–

S, syngeneic; A, allogeneic; 21.x, rat number x twenty-onedays after transplantation; Duration of surgical procedures are given in minutes; -, none; +, mild; ++, severe.

Skin transplantation. At day 7, all 3 WAG skin grafts on DA recipients developed local erythema and edema, which resembles ongoing rejection by their DA recipients. At day 14, 100% necrosis was seen and the entire surface of the grafts were hardened and escharified with hair loss. This was defined as complete rejection.

Cell viability. The SI of all PBMC and spleen populations surpassed 25 after 72h of incubation with the mitogen Con A, indicating that the cells used in the LDA were viable.

HTLf determination. The pretransplantation frequency of HTL in peripheral blood of DA rats was evaluated using limiting dilution analysis towards WAG antigens. The median pretransplant HTLf against WAG was $17/10^6$ (range 4-$19/10^6$ PBMC). Seven days after aortic valve implantation the median donor reactive HTLf in peripheral blood of allogeneic transplanted rats was $26/10^6$ PBMC(range:10-$39/10^6$ PBMC), not significantly different from the syngeneic ($19/10^6$ PBMC (range: 4 -$24/10^6$ PBMC), p=0.17) or pretransplant HTLf ($17/10^6$ PBMC, p=0.16).
Twenty-one days after aortic valve transplantation, the median HTLf in the allogeneic group was $109/10^6$ PBMC (range:87-122 $/10^6$ PBMC)and in the syngeneic group $17/10^6$ PBMC (range:11-$57/10^6$ PBMC) in peripheral blood, considered significantly different (p=0.004).
Also, the increase compared to the pretransplant HTLf was significantly higher in the allograft recipients after 21 days (p=0.007) (Fig 3).

Figure 3

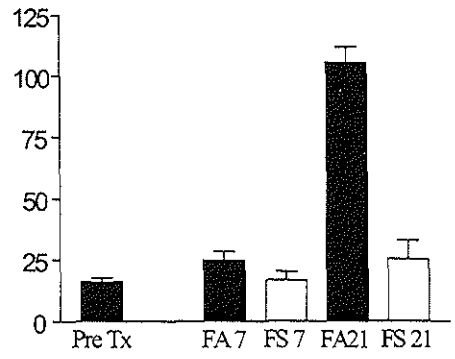


Figure 4

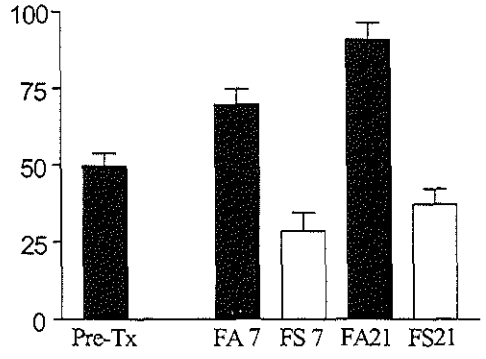


Figure. 3. shows the frequencies of WAG-directed HTL in peripheral blood (Y-axes) of aortic valve recipients (DA) compared to pre-transplantation frequencies (pre Tx). FA fresh allogeneic valve graft, FS fresh syngeneic valve graft, 7 and 21 days after transplantation. Note the significant increase (p=0.007) of HTLf 21 days after FA transplantation compared to pre-transplantation frequencies and FS 21.

Figure. 4. shows the frequencies of WAG-directed HTL in the spleen (Y-axes) of aortic valve recipients (DA) compared to frequencies of non-transplanted DA-rats (non-Tx). FA fresh allogeneic valve graft, FS fresh syngeneic valve graft, 7 and 21 days after transplantation. HTLf of FA transplanted rats at 7 and 21 days differed significantly from non-transplanted and FS counterparts.

In spleens of rats receiving an allogeneic valve, a median HTLf of 71/10^6 spleen cells (range: 54-84/10^6 spleen cells) and 24/10^6 spleen cells (range: 17-51 /10^6 spleen cells) in syngeneic graft recipients was measured on postoperative day 7. This difference is statistically significant (p=0.008). At 21 days, the median HTLf in the allogeneic transplanted rats was 92/10^6 spleen cells (range:75-109/10^6 spleen cells), and in the syngeneic group 34/ 10^6 spleen cells (range:28-55/10^6 spleen cells), considered significant (p=0.008). HTLf in the allogeneic group differed significantly from frequencies measured in non-transplanted rats (p=0.02) (Fig 4)

Seven days after skin grafting, HTLf against WAG-antigens in peripheral blood were increased by a factor 4.3 compared to the pre-transplant frequency (p=0.028). Fourteen days after skin transplantation, the day of complete graft rejection, the peripheral blood HTLf were 5.7 times higher as before transplantation (p=0.01). At day 21, circulating donor-reactive HTLf were normalised to the pretransplant value. In the spleen, HTLf could only be measured after 21 days, the day of sacrifice. At that time, the immune response was already extinguished and a median HTLf of 15/10^6 spleen cells (range: 6-20/ 10^6 spleen cells) was found. In valve recipients, the increase factors were 1, 1.5 and 1.5 for the syngeneic and 1.5, 2.6 and 6.3 for the allogeneic valve recipients respectively (Fig 5).

Figure 5

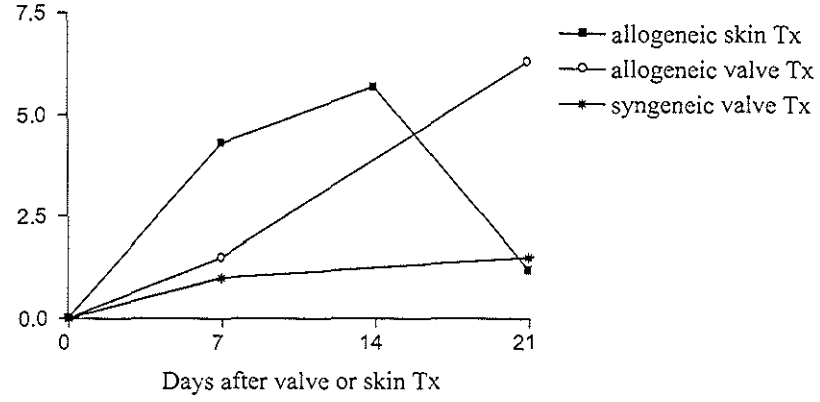


Figure. 5. demonstrates the ratio of HTLf (post/pre-transplantation) measured in peripheral blood (Y-axes) of rats 7, (14) and 21 days after skin or valve transplantation. Maximal HTLf after skin transplantation were detected earlier than after valve transplantation. Immune reactivity was extinguished at 21 days after skin transplantation and not after allogeneic valve transplantation, suggesting different pathways of rejection after allogeneic skin and valve transplantation.

Discussion

In clinical heart valve transplantation, the effect of allo-reactivity on structural deterioriation and dysfunction of the valve allograft is still a matter of debate. Therefore, various *in vivo* rat models have been developed to study the immune response after valve transplantation using a variety of MHC-disparent inbred rat strains (14,15). Most models are biased by technical inadequacies. The "Rotterdam" model is suitable to investigate immunological causes of heart valve deterioration in an accurate and technically unbiased manner (11). In clinical heart valve transplantation, an objective parameter for the detection of graft rejection after transplantation, such as endomyocardial biopsy in cardiac transplantation, is absent. Therefore, other methods are required. The increase of circulating donor-reactive HTLf during histological proven cardiac allograft rejection demonstrates the importance of HTLs directed against allo-antigens in the immunological effector mechanisms that underly graft rejection (10). Therefore, measuring these cells might be useful to indicate valve allograft rejection. Bishop *et al.* demonstrated that IL-2 producing HTLs are absolutely required in rejection processes (16).

In the present study, we examined the role of donor-reactive HTL in valve allograft rejection using a strong allogeneic inbred rat strain combination. Seven days after fresh aortic valve transplantation, no increase was found in HTLf in peripheral blood of allogeneic or syngeneic transplanted animals, whereas in the spleen higher HTLf was found in allograft recipients compared to pre-transplant frequencies ($p=0.06$) and syngeneic controls ($p=0.008$). Twenty-one days after implantation, a major increase of HTLf against WAG antigens in peripheral blood was found in allograft recipients compared to pre-transplantation frequencies ($p=0.008$) and syngeneic transplanted rats ($p=0.004$). The HTLf after valve allograft implantation was comparable to that induced by a skin allograft from the same donor strain. However, because of its high immunogenicity maximal reactivity after skin transplantation was detected at an earlier time point. This can be explained by a higher density of Antigen Presenting Cells (APC, Langerhans Cells) in the skin compared to the valve implants (Dendritic Cells). In the spleen of the allogeneic group HTLf were also elevated but again not in the syngeneic group.The relatively late increase of HTLf in peripheral blood compared to HTLf in the spleen may have two explanations. First, a substantial number of HTL could have left the circulation at day 7 after implantation by infiltration into the valve allograft. By day 21, when a major part of the alloantigens in the graft has been vanished, activated HTLs do not enter the circulation anymore. Our histological findings of massive infiltration of the valve leaflets and vascular wall already at day 7, leaving the valve leaflets non-cellular and deformed by day 21, support this theory. Secondly, during the early phase of the allo-response, the majority of HTLs is still in the secondary lymphoid organs, such as the spleen and peripheral lymph nodes . No dysfunction of valve allografts was seen at seven days postoperatively. This is in compliance with histological analysis, where infiltration of the allografts was present, but no signs of structural changes of the valve leaflets.

Histological evaluation of the allograft at 21 days postoperatively revealed infiltration of mononuclear cells of the graft, as well as valve deformation. This histopathology corresponds with rejection, also described by other groups (17,18). The microscopical aspect of syngeneic grafts was normal at all time-points. Valve function at 21 days after the operation showed incompetence in the allogeneic situation, in contrast to the syngeneic valves, where none of the 5 valves were incompetent. The acellularity of the valve leaflets 21 days postoperatively is likely to be immunologically mediated, because of the presence of severe infiltrations of the graft seen at 7 days post-transplantation. HTLf measured in peripheral blood after valve transplantation corresponds with signs of rejection 21 days post-transplantation, confirmed by HTLf during rejection of skin grafts. In spleen, HTLf against WAG are elevated earlier, already 7 days after transplantation, where histology revealed infiltration of mononuclear cells but no valve destruction and no valve dysfunction. This leads to the conclusion that HTLf measured in peripheral blood corresponds with rejection of fresh aortic valve allografts in rats.

Before extrapolating these results to the clinical situation, one has to bear in mind that this study was performed with animals receiving a fresh aortic valve grafts, while in clinical practice cryopreserved valve allografts are frequently used. Despite this relative disadvantage, the data of this study show that immunological sensitization can have clinical importance regarding long-term valve allograft function. Ex vitro monitoring of donor-reactive HTL frequencies in the peripheral blood of allograft recipients could be a useful tool in monitoring immune activity in patients after implantation of human cardiac valves in order to diagnose rejection.

References

1. O'Brien, M.F., Stafford, E.G. and Gardner, M.A. Allograft aortic valve replacement: Long-term follow-up. *Ann Thorac Surg* 60:S65, 1995.
2. Niwaya, K., Knott-Craig, C.J., Lane, M.M., Elkins, R.C. et al. Cryopreserved homograft valves in the pulmonary position: Risk analysis for intermediate-term failure. *J Thorac Cardiovasc Surg* 117:141, 1999.
3. Willems, T.P., Bogers, A.J.J.C., Cromme-Dijkhuis, A.H. et al. Allograft reconstruction of the right ventricular outflow tract. *Eur J Cardio-Thorac Surg* 10:609, 1996.
4. Baskett, R.J., Ross, D.B., Nanton, M.A. and Murphy, D.A. Factors in the early failure of cryopreserved homograft pulmonary valves in children: preserved immunogenicity? *J Thorac Cardiovasc Surg* 112: 1170,1996.
5. Rajani, B., Mee, R.B. and Ratcliff, N.B. Evidence for rejection of homograft cardiac valves in infants. *J Thorac Cardiovasc Surg* 115:111, 1998.
6. Hoekstra, F.M.E., Knoop, C.J., Vaessen, L.M.B. et al. Donor-specific immune response against human cardiac valve allografts. *J Thorac Cardiovasc Surg* 112:281, 1996.
7. Hogan, P.G., Duplock, L., Green, M.K., Smith, S., Gall, K.L., Frazer, I.H. and O'Brien, M.F. Human aortic valve allografts elicit a donor-specific immune response. *J Thorac Cardiovasc Surg* 112:1260, 1996.
8. Hall B.M. Cells mediating allograft rejection. *Transplantation* 51:1141, 1991.
9. Debruyne, L.A., Ensley, R., Olsen, S.L.,Taylor, D.O., Carpenter, B.M., Holland, C, Swanson, S., Jones, K.W., Karwande, S.V., Renlund, D.G. and Bishop, D.K. Increased frequency of alloantigen-reactive helper T lymphocytes is associated with human cardiac allograft rejection. *Transplantation* 56:722, 1993.
10. Vaessen, L.M.B., Daane, C.R., Maat, A.P.W.M., Balk, A.H.M.M., Claas, F.H.J. and Weimar, W. T helper frequencies in peripheral blood reflect donor-directed reactivity in the graft after clinical heart transplantation. *Clin Exp Immunol* 118:473, 1999.
11. Oei, F.B.S., Welters, M.J.P., Bonthuis, F., Vaessen, L.M.B., Marquet, R.L., Weimar, W. and Bogers, A.J.J.C. A size-matching heterotopic aortic valve implantation model in the rat. *J Surg Res* 87:239, 1999.
12. Deadock, S., Schwarer, A., Batchelor, R., Goldman, J. and Lechler, R. A rapid limiting dilution assay for measuring frequencies of alloreactive, interleukin-2-producing T cells in humans. *J Immunol Methods* 147:83, 1992.
13. Strijbosch, L.W.G., Buurman, W.A., Does, R.J.M.M. et al. Limiting dilution analysis. Experimental design and statistical analysis. *J Immunol Methods* 197:133, 1987.
14. Yankah, A.C., Dreyer, W., Wottage, H.U. and Muller, H.W. Kinetics of endothelial cells of preserved aortic valve allografts used for heterotopic transplantation in inbred rat strains. In Bodnar E. and Yacoub M.H. (eds.). *Biologic and Bioprosthetic Valves*. New York: Yorke Medical Books, 1986, Pp. 73-87.
15. Zhao, X., Green, M.K., Frazer, I.H., Hogan, P. and O'Brien, M.F. Donor-specific immune response after aortic valve allografting in the rat. *Ann Thorac Surg* 57:1158, 1994.
16. Bishop, D.K., Shelby, J. and Eichwald, E.J. Mobilization of T lymphocytes following cardiac transplantation: evidence that CD4-positive cells are required for cytotoxic T lymphocyte activation, inflammatory endothelial development, graft infiltration, and acute allograft rejection. *Transplantation* 53:849, 1992.
17. Moustapha, A., Ross, D.B., Bittira, B. et al. Aortic valve grafts in the rat: evidence for rejection. *J Thorac Cardiovasc Surg* 114: 891, 1997.
18. Green, M.K., Walsh, M.D., Dare, A. et al. Histologic and immunohistochemical responses after aortic valve allografts in the rat. *Ann Thorac Surg* 66: S216, 1998.

CHAPTER 10

Immunological aspects of fresh and cryopreserved aortic valve transplantation in rats

F.B.S. Oei, A.P.A. Stegmann, L.M.B. Vaessen, R. L. Marquet, W. Weimar and A.J.JC. Bogers.

Published in Annals of Thoracic Surgery, 2001; 71:S 379-84

Abstract

The influence of immune activation on valve allograft degeneration remains unclear. We studied the combined effect of major histocompatibility complex (MHC-)-incompatibility and cryopreservation on valve performance, histomorphology, and tissue antigenicity in rats.

Fresh or cryopreserved allogeneic aortic valves from WAG ($RT1^u$) rats were transplanted to DA ($RT1^a$) recipients and syngeneic transplants served as controls. After 7 or 21 days, valves were examined for competence and morphology. Immune reactivity of the recipient was measured by concanavalin A (ConA) stimulation and analysis of donor-reactive Helper T-lymphocyte frequencies (HTLf) in peripheral blood and spleen.

Syngeneic grafts demonstrated normal competence and structure. Allografts lost their competence over time caused by destruction of the leaflets combined with cellular infiltration in the vascular wall. Cryopreservation induces early loss of competence and retrovalvular thrombosis. Cryopreserved allografts were also heavily infiltrated. ConA stimulation indices and HTLf were higher in allogeneic recipients compared to syngeneic recipients ($p < 0.03$). Cryopreserved allografts elicited a lower immune response compare to fresh allografts ($p < 0.03$).

Aortic valve allografts are able to induce a donor-reactive immune response, that is related to early graft destruction and incompetence. Cryopreservation appears to diminish but not eliminate the immunogenicity of the allograft.

Introduction

Theoretically, valve allograft failure may be due to immunological factors induced by blood group (ABO) or human leukocyte antigens (HLA) mismatch. Previous *in vivo* studies already demonstrated the immunogenicity of human valve allografts [1], later confirmed by clinical *ex vivo* studies [2]. The method of cryopreservation, introduced by O'Brien *et al*, improves long-term graft performance by retaining the viability of valvular cells [3]. Nevertheless, viable valvular cells could express HLA antigens, which in HLA mismatched condition could trigger an allogeneic response leading to graft rejection. Echocardiographic monitoring of the aortic valve allograft in the patient can be useful for monitoring the valve function. For diagnosis of graft rejection, which is characterized by cellular infiltration and tissue necrosis, continuous histopathologic analysis of the transplanted tissue is necessary. This is certainly not possible in human valve allograft follow-up. Several in vivo studies, using inbred rat strains, have brought evidence for immune-mediated structural damage in fresh valve allografts [4]. However, the influence of cryopreservation in this process remains unclear.
In this article we describe the functional, pathologic and immunologic changes of aortic valve transplantation, by using an 'end-to-side' heterotopic implantation model in rats with strong histo-incompatibility [5]. In addition, the effect of cryopreservation on valve immunogenicity and structure was studied.

Materials and Methods

Experimental design. This study includes four experimental groups consisting of fresh allogeneic (FA) and cryopreserved allogeneic (CA) and fresh syngeneic (FS) and cryopreserved syngeneic (CS) aortic valve transplantations. Male inbred DA (RT-1^a/RyHSD) and WAG-Rij (RT-1^u/RyHSD) rats, weighing 200 to 250 g (Harlan CPB, Horst, The Netherlands), were used as recipients or syngeneic donors and allogeneic donors respectively. A modified intraabdominal implantation model was used [5]. Each group included 12 animals, sacrificed either at day 7 (n=6) or day 21 (n=6) followed by extraction of peripheral blood, spleen and valve graft. After gross examination of the graft, valve competence was analyzed by retrograde injection of saline. Subsequently, standard histologic and immunohistochemical evaluations were performed on all explanted grafts. Immune reactivity was measured among peripheral blood mononuclear cells (PBMC) and spleen cells using concanavalin A (ConA) stimulation and donor-reactive T-lymphocyte frequency (HTLf) analysis. Surgical failures including ischemic or neurologic complications, preliminary death, obliteration of the graft and aneurysm of the anastomosis were excluded from the study. The experimental protocols were concordant with the Guidelines on the Protection of Experimental animals by the Council of the European Community (1986) and approved by the Committee on Animal Research of Erasmus University of Rotterdam, The Netherlands.
Aortic valve grafts were directly implanted or transported to the Heart Valve Bank Rotterdam after procurement for sterilization, cryopreservation and storage (at least 4 weeks) conform the standard protocol [2]. Before implantation the valves were thawed in a 37°C water bath and gently flushed with heparinized saline (50 U/ml). Before implantation valve grafts were checked for competence by retrograde injection with saline.

Histological analysis. After competence testing and macroscopic examination of the leaflet, sinus of Valsalva and vascular wall, the explanted grafts were dissected longitudinally into three symmetric rectangular pieces for standard histologic and immunohistochemical staining. Valve pieces were fixed in 10% buffered formaldehyde for at least 24h and embedded in paraffin. Hematoxylin-eosin stained 4 μm sections were examined under microscope by 2 independent investigators (F.B.S.O, A.P.A.S). The presence of mononuclear cells infiltration in all three vascular wall layers and valve leaflet was scored semiquantitatively (grade 0 = normal, 1 = mild, 2 = moderate and 3 = severe). For phenotyping the infiltrates, immunohistochemical staining of frozen valve sections (5 μm) was performed by three-layer immunoperoxidase technique [5]. We used 5 different mouse-anti-rat IgG monoclonal primary antibodies (Serotec Europe, Oxford, UK) directed against epitopes present on specific cells shown in table II. The number of positive-stained cells in 3 consecutive areas of the valve grafts (leaflet, hinge area and vascular wall) were scored by using an ocular grid allowing cell counts per 0.1 mm^2. Analysis of non-specific tissue reactions near the sutures was avoided.

ConA stimulation and HTLf analysis. Peripheral blood mononuclear cells were isolated from the peripheral blood by Ficoll-Hypaque (Amersham Biotech AB, Uppsala, Sweden) density gradient and immediately used for in vitro tests. Recipient or donor spleen cells were obtained by mechanical dissociation of the spleen and Ficoll-Hypaque density gradient.

Peripheral blood mononuclear cells and spleen cells from all recipient animals were used for ConA stimulation tests. Triplicate cultures of 10^5 PBMC or 5.10^4 spleen cells were incubated for 72 hours at 37°C and 5% CO_2 in 96-well, round-bottomed microtiter plates (Nunclon, Roskilde, Denmark) containing culture medium (RPMI 1640 supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES solution, 5.10^{-5} mol/L 2-mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% heat inactivated fetal bovine serum) with or without 10 mg/ml of ConA (Amersham). To each well, 0.5 μCi of ^{3}H-thymidine (Amersham) was added 8 hours before harvesting. Incorporated ^{3}H-Thymidine was measured in a scintillation spectrophotometer (Betaplate 1205; LKB-Wallac, Turku, Finland). The stimulation index (SI) was calculated by the formula: SI= mean counts per minute (cpm) with ConA divided by mean cpm without ConA.

Helper T lymphocyte frequencies were calculated by limiting dilution assay of PBMC and spleen cells from each experimental animal. Twelve replicate cultures were set up for six dilutions of responder cells (PBMC: 1.10^5 to 1563 per well; spleen cells: 5.10^4 to 781 per well), from recipients origin, in 96-well U-bottomed microtiter plates containing 5.10^4 per well irradiated (30 Gy) splenic donor or autologous cells. After incubation in culture medium for 72 hours at 37°C and 5% CO_2, 100 μl supernatant of each well was transferred to new U-bottomed plates. Functional interleukin-2 (IL-2) in the supernatant, produced by the responding HTLfs, were measured and calculated using an IL-2 bioassay [6]. Frequency estimates are presented as the number of helper T-lymphocytes per million PBMC or spleen cells [6].

Statistical analysis. Differences between the experimental groups were tested with Mann-Whitney *U*-test using InStat software (GraphPad, San Diego, CA). The limit of significance was set on a two-tailed *p*-value less than 0.05.

Results

Functional and macroscopic evaluation. Three animals were excluded from the study because of death by hemorrhage or obliteration of the graft. Syngeneic grafts all remained competent after transplantation, while fresh allogeneic valve grafts progressively lost their competence. Early valve incompetence was observed in the cryopreservation groups. Thickening of valve leaflet and aortic wall and sinus thrombosis was only seen among allografts (Table I).

Table I: Valve competence and histo-morphology of aortic valve grafts

	Valve incompetence‡	Retrovalvular thrombosis	Thickening leaflet / wall	Infiltration intima*	Infiltration media*	Infiltration adventitia*	Infiltration valve leaflet*
FS 7d (n=5)	0%	0%	20%	0	1	1	0-1
FS 21d (n=6)	0%	0%	17%	0-1	0-1	0-1	0
FA 7d (n=6)	17%	33%	100%	0-1	1	2	2-3
FA 21d (n=6)	100%	50%	100%	1-2	1-2	3	0
CS 7d (n=5)	60%	40%	60%	0-1	0-1	1	0-1
CS 21d (n=6)	83%	67%	67%	0-1	0-1	0-1	0
CA 7d (n=5)	100%	100%	100%	0-1	0-1	2	1-2
CA 21d (n=6)	100%	100%	100%	1-2	1	3	0-1

*FS: Fresh syngeneic, FA: Fresh allogeneic, CS: Cryopreserved syngeneic, CA: Cryopreserved allogeneic, 7d/21d: seven and twenty one days post transplantation; (n): number of grafts in category; ‡: percentage of affected valves; *: semi-quantitative scoring: 0: normal, 1: mild, 2: moderate, 3: severe.*

Histology and Immunohistochemistry. Syngeneic grafts demonstrated minor infiltration of mononuclear cells in the leaflet and adventitial layer at day 7 days that was diminished by day 21. Progressive (moderate to severe) infiltration of ($CD8^+$) T cells, macrophages and dendritic cells was prominent in the adventitial layer spreading to the medial and intimal layer of allografts. Allogeneic valve leaflets were infiltrated by ($CD8^+$) T cells and macrophages leading to complete loss of stromal cells and distorted leaflet structure at day 21 (Fig 1A, 1B). Cryopreserved syngenic grafts showed prolonged influx of T cells and macrophages, while cryopreserved allografts were infiltrated by mononuclear cells similar to their fresh counterparts. Progressive reduction of the numbers of cells stained positively for donor MHC class II antigens was observed in the valve leaflet and the hinge area of allografts (Fig 2A, 2B). Cryopreservation appeared to decrease the amount of donor derived cells (Fig 2C) (See tables 1 and 2).

Figure 1

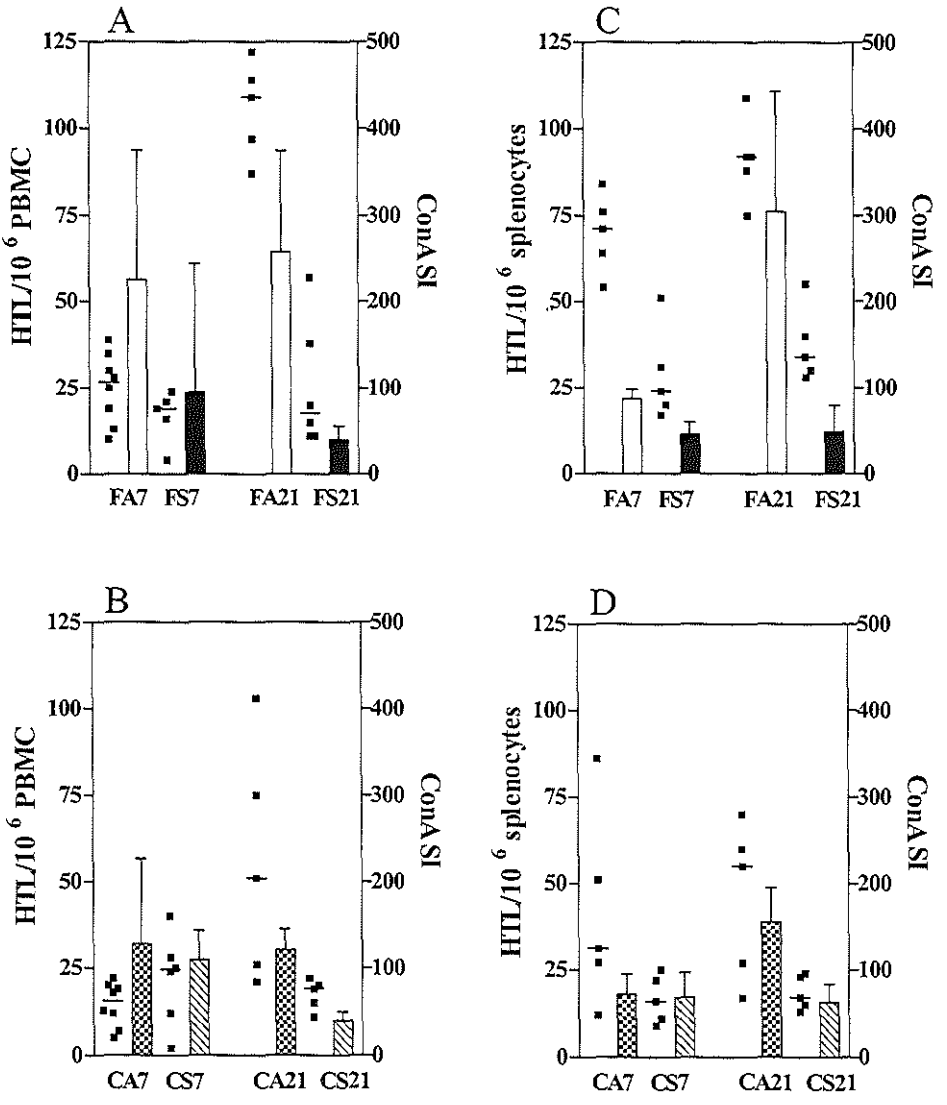


Figure 1: Frequency of donor-reactive Helper T lymphocytes (left scatters, left Y-axes) and concanavalin A stimulation indices (right bar, right Y-axes) among peripheral blood lymphocytes (A+B) or spleen cells (C+D) of different aortic valve recipients at different time points.(FA: fresh allograft, CA: cryopreserved allograft, FS: fresh syngeneic graft, CS: cryopreserved syngeneic graft, 7: seventh post transplantation day; 21: twenty first post transplantation day). (Horizontal) bars resemble median value and range.

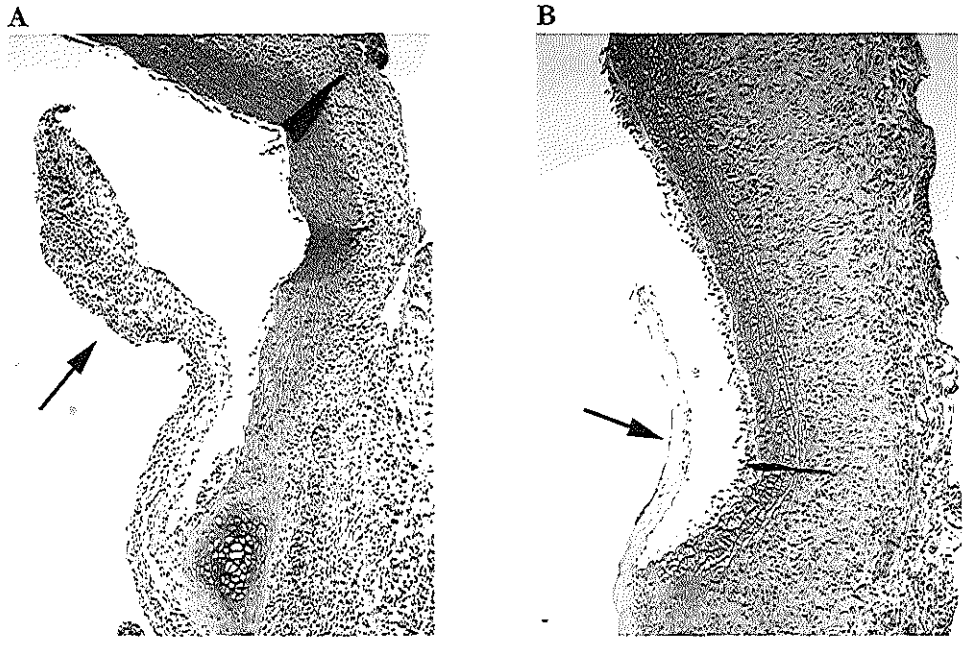


Figure 2: Light-microscopical photographs of longitudinal sections of fresh aortic valve allograft (WAG to DA) at 7 (A) and 21 (B) days after implantation (100×, hematoxylin-eosin stained). Valve leaflet (arrow) is heavily infiltrated by mononuclear cells at day 7 which resulted in dysmorph, acellular leaflets at day 21. Adventitial wall thickening, infiltration and media cell loss was progressive in time.

Table II: Immunohistochemical analysis of aortic valve graft

	R73 (pan T cell)	OX8 (CD 8⁺ T-cell)	ED1 (Macrophage)	OX62 (Dendritic cell)	OX3 (Donor MHC class II)
FS 7d (n=5)	13 (1-19)	6 (0-15)	21 (6-59)	5 (2-10)	-
FS 21d (n=6)	9 (7-35)	7 (3-8)	11 (6-39)	7 (4-15)	-
FA 7d (n=4)	31 (16-109)	23 (14-79)	28 (23-56)	17 (6-29)	32 (1-89)
FA 21d (n=5)	39 (18-66)	29 (7-80)	71 (20-89)	14 (8-23)	5 (0-19)
CS 7d (n=4)	13 (5-23)	11 (4-18)	21 (11-37)	5 (3-7)	-
CS 21d (n=5)	13 (7-22)	13 (2-21)	20 (8-45)	6 (2-8)	-
CA 7d (n=4)	48 (39-55)	36 (27-43)	37 (26-61)	16 (15-20)	19 (3-56)
CA 21d (n=5)	47 (20-57)	31 (15-51)	47 (11-59)	15 (9-18)	4 (3-17)
Control (n=5)	4 (3-7)	3 (1-6)	5 (4-6)	3 (1-4)	-

Grafts coded as in Table I. Five different monoclonal antibodies were used to identify specific cells. Figures represent the median numbers and the range(.) of positive stained cells per 0.1 mm² as average of three graft areas (leaflet, hinge area and vascular wall); Control: non-implanted DA valves.

Concanavalin A stimulation and HTLf. ConA stimulation (Fig 3) indices of all cell samples exceeded 25, which indicated the viability and immune reactivity of the cell samples. At day 21, spleen cells from allograft recipients had a higher SI compared to syngeneic graft recipients ($p < 0.01$) whereas cryopreservation of the graft reduced the reactivity of graft recipients (CA<FA). In allograft recipients there was progressive increased reactivity among PBMC ($P < 0.01$). By day 21, allograft recipients showed elevated SIs within PBMC again, whereas the SIs of isograft recipients returned to their pre-operative value (pre-op median SI =40.0, $p < 0.002$).

Helper T lymphocyte frequencies (HTLf) (Fig 3) were higher among spleen cells of allograft recipients compared to isograft recipients at both days 7 and 21 ($p < 0.05$). In peripheral blood, increased HTLf was only seen at day 21 among allografts recipients. Recipients of fresh allografts gained higher frequencies compared to cryopreserved allografts ($p < 0.05$).

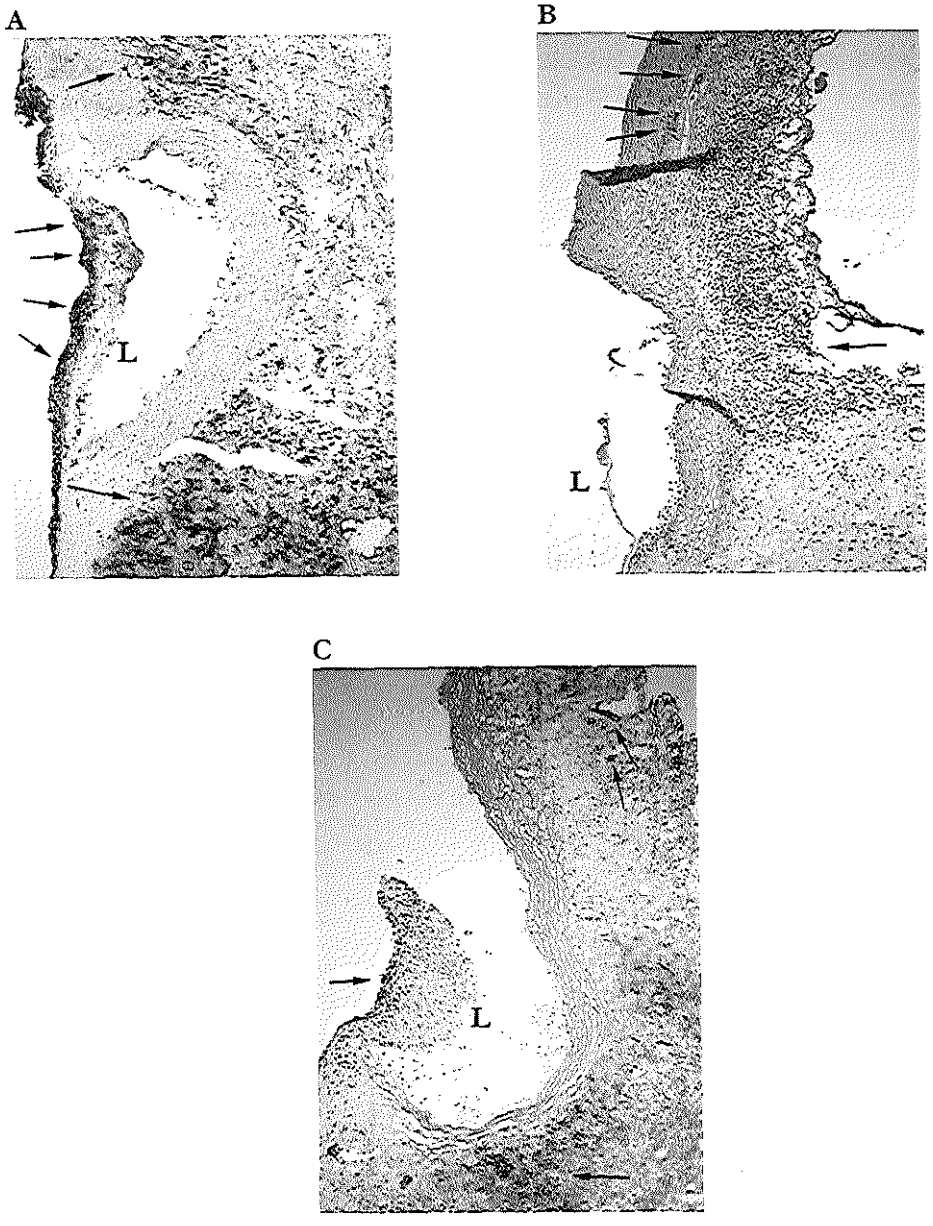


Figure 3: Light-microscopical photographs of immunohistochemical stained (anti-OX3, WAG MHC class II antigen) sections of fresh allogeneic valve grafts (WAG to DA) at 7 (A) and 21 (B) days after implantation (100×). Reduction of areas consisting cells positive for OX3 is seen in time (arrows), indicating loss of donor derived MHC class II positive cells. (C) OX3 expression (arrow) of cryopreserved valve allograft at day 7 after implantation illustrating early reduction of donor derived cells compared to fresh counterparts. (L): valve leaflet.

Discussion

The results presented in this study demonstrate that fresh aortic valve allografts implanted heterotopically in rats exhibit early histopathological changes characteristic for rejection. Our findings are in agreement with other comparable studies suggesting that immune-mediated processes cause valve allograft deterioration [4, 7]. Interestingly, intense and progressive infiltration of T lymphocytes, macrophages and dendritic cells in the allogeneic valve leaflets (day 7) did not result in valve dysfunction. Valve incompetence was found later at day 21 and was accompanied by acellular and structural distorted leaflets, illustrating that early cellular infiltration can lead to structural valve deterioration and dysfunction. Phenotyping the infiltrates revealed early involvement of $CD8^+$ T lymphocytes suggesting T cells to be the primarily effector cells initiating and regulating the donor-specific immune response. The influx of macrophages in the allograft is progressive and could be stimulated by local expression of cytokines and chemokines by activated T-cells. It was found that the number of dendritic cells had increased in allografts, whereas only a few donor major histocompatability complex class II positive cells were seen. We suggest that the dendritic cells, involved in this allo-response are recruited from the recipient.

The histo-morphologic changes of allografts are accompanied by an anti-donor immune response detected in the spleen and peripheral blood. T-cell reactivity measured by ConA stimulation revealed early increase of SI among PBMCs probably caused by a nonspecific activation after surgical intervention. At day 21 the SI of syngeneic graft recipients returned to normal value (SI=40), whereas the value remained high in allograft recipients because of persistent allostimulation. Among the splenocytes, a similar difference in SI was seen, which suggest an increase of activated T cells in the secondary lymphoid organs. The analysis of Helper T lymphocyte frequencies (HTLf) in the spleen demonstrates increased allo-reactive HTLf among splenocytes already at day 7. These findings correspond with the study performed by Zhao and colleagues, who also identified an increase of donor specific cytotoxic T cells after valve allograft implantation in rats [7]. However, their experiments did not include analysis of immune competent cells the in peripheral circulation. In our study, donor-reactive HTLf increase among PBMCs were observed at a later time point, indicating a sequence of primary activation of HTL in lymphoid organs followed by secondary release of activated HTL in the peripheral blood.

Histopathological differences between cryopreserved syngeneic and allogeneic valve grafts were comparable to their fresh counterparts. However, sinus thrombosis, medial cell loss and intima thickening were more prominent in the cryopreserved valves and these were caused by the cytotoxic effect and physical surface damage by cryopreservation [4, 8]. In addition, cryopreservation of the allografts appeared to reduce the immunogenicity of valve grafts, shown by a significant lower ConA reactivity and HTLfs in both spleen and PBMCs compared fresh allograft recipients ($p < 0.03$). Nevertheless, cryopreserved allografts maintain their capacity to induce an immune response within

the recipients indicated by higher anti-donor T-cell reactivity compared to syngeneic graft recipients ($p<0.03$).

In this study we have demonstrate that valve allografts are able to provoke a donor-reactive immune response within the recipient resulting in graft destruction and dysfunction. Cryopreservation of valve allografts not only diminishes the immunogenicity but they also alter their structural nature leading to additional nonimmunological degeneration. The increase of HTLf in both spleen and peripheral blood after allogeneic transplantation corresponds with histopathologic signs of rejection in the graft and therefore confirms the important role of helper T cells in the immune response underlying allograft rejection [9]. Therefore, we suggest that clinical monitoring of helper T cell frequencies in peripheral blood of valve allograft recipients could serve as a non-invasive tool for identifying cellular rejection of the transplanted valve.

References

1. Hoekstra F, Knoop C, Jutte N. *et al.* Effect of cryopreservation and HLA-DR matching on the cellular immunogenicity of human cardiac valve allografts. J Heart Lung Transplant 1994; 13(6): 1095-8
2. Oei FB, Welters MJ, Vaessen LM, Stegmann AP, Bogers AJ, Weimar W. Induction of cytotoxic T lymphocytes with destructive potential after cardiac valve homograft implantation. J Heart Valve Dis 2000; 9:761-8
3. O'Brien MF, Stafford EG, Gardner MA, Pohlner PG, McGiffin DC. A comparison of aortic valve replacement with viable cryopreserved and fresh allograft valves, with a note on chromosomal studies. J Thorac Cardiovasc Surg 1987; 94(6): 812-23
4. Moustapha A, Ross DB, Bittira B *et al.* Aortic valve grafts in the rat: evidence for rejection. J Thorac Cardiovasc Surg 1997; 114: 891-902
5. Oei FB, Welters MJ, Vaessen LM, Marquet RL, Weimar W, Bogers AJ. Heart valve dysfunction due to cellular rejection in a novel heterotopic transplantation rat model. Transpl Int 2000;13:S528-31
6. Vaessen LM, Daane CR., Maat AP, Balk AH, Claas FH and Weimar W. T helper frequencies in peripheral blood reflect donor-directed reactivity in the graft after clinical heart transplantation. Clin Exp Immunol 1999; 118: 473-9
7. Zhao XM, Green M, Frazer IH, Hogan P, O'Brien MF. Donor-specific immune response after aortic valve allografting in the rat. Ann Thorac Surg 1994; 57: 1158-63
8. Legare JF, Lee TD, Ross DB. Cryopreservation of rat aortic valves results in increased structural failure. Circulation 2000;102:SIII75-8
9. Hall BM. Cells mediating allograft rejection. Transplantation 1991; 51: 1141-51

PART III

DISCUSSION AND SUMMARY

CHAPTER 11

General Discussion & Conclusions

F.B.S Oei

Introduction

Human valve allograft is an alternative to other biological cardiac prostheses available for surgical management of acquired and congenital heart diseases. The advantages of human donor valves are obvious and include superior hemodynamical properties especially among small sizes used for surgical reconstruction in young patients with congenital heart defects. Other important advantages include the lack of post-operative thrombo-embolic complications, which prevents anti-coagulation of the valve recipient. The relative resistance to endocarditis also makes donor valves the favorite prostheses in endocarditis related replacements [1]. However, the availability and limited durability of human valve allografts are the most important drawbacks limiting the universal use of these bio-prostheses. The availability of valve allografts has increased progressively thanks to major improvements made in tissue preservation techniques. The basic issue in valve allograft storage has been the preservation of the cellular viability and the structural integrity of the leaflets. Meanwhile, the association between tissue viability and allograft durability remains a major subject of discussion [2]. On the other hand, increased cellular viability may also result in the enhancement of graft immunogenicity [3].

Clinical follow-up studies regarding valve allograft implantation successively reported good mid-term results. Therefore, donor valve prostheses are considered as tissue grafts with low immunogenicity, which can be transplanted without matching of ABO blood group or tissue antigens like Human Leukocyte Antigens (HLA). Consequently, unlike solid organ or bone marrow recipients, immunosuppressive drugs preventing severe graft rejection, are not routinely administered to valve allograft recipients. During the last decade, increasing evidence of valve allograft inducing immune activation is provided by clinical and experimental animal studies. Several investigators who found mononuclear cell infiltration in explanted dysfunctional grafts suggested an important association between tissue immunogenicity and immune mediated valve deterioration [3-6]. In contrast, this theory is refuted by others claiming that the structural integrity, which is more related to the method of preservation and therefore may be the crucial factor for valve allograft durability [7, 8]. Altogether, the relative effect of allo-activation by heart valve transplants in valve destruction is still unresolved.

In this thesis we have investigated the immunological aspects of donor valve transplantation by analyzing the cellular antigenicity of fresh and cryopreserved semilunar valves. Secondly, by monitoring the donor-specific responsiveness of circulating T-lymphocytes in valve recipients, we studied the immunogenicity of clinically transplanted valve allografts and the ability of the recipient to mount a specific response against the allogeneic valve. Thirdly, we investigated the relation between immune activation, structural degeneration and dysfunction of fresh and cryopreserved aortic valve allografts by using a heterotopic aortic valve transplantation model in rats.

Immunological aspects of valve allograft implantation: Clinical studies

In the study described in chapter 2, we found that the position of the allograft (anatomical or extra-anatomical) and the nature of the valve (aortic or pulmonary) are risk factors for accelerated allograft failure. In line with the result of earlier studies, mismatch of blood group antigens (ABO) appeared not to be a relevant factor in the process of valve deterioration [9, 10]. On the other hand, in organ and bone marrow transplantation, matching of ABO-antigens between donors and recipients remains crucial for preventing acute rejection. Our observation may imply that donor cardiac valves lack expression of ABO-antigens. This assumption has been confirmed recently in a histological study performed by Kadner and colleagues [11]. In their study, no ABO-antigen expression was found on the luminal endothelial cells of human cardiac valves, while endothelium from human umbilical cord, saphenous vein and myocardium were ABO-positive. Besides ABO-antigens, Human Leukocyte Antigens (HLA) are prerequisite for the induction of specific immune responses against allogeneic tissue. HLA class II molecules (DR, DQ and DP) can be expressed by antigen presenting cells (APC). These APCs are able to directly present allogeneic antigens to immune competent cells of the recipient and therefore play a major role in activation of the immune system [12]. Studies regarding the effect of HLA (especially class II) mismatching in valve allograft survival are scarce. Recently, Dignan and colleagues demonstrated in a retrospective study that mismatch of HLA class II molecules was associated with a significantly lower freedom from structural deterioration of cryopreserved valve allografts [13]. In a comparable study, Smith and colleagues did not find a correlation between HLA mismatch and failure of valve allografts stored in antibiotic solution at 4°C [14]. The difference in results may be based on differences in immunogenicity of cryo- or antibiotic (4°C) preserved valves. Viability studies have demonstrated that the cellular components of valve allografts preserved at 4°C in antibiotic solutions are less viable when compared to cryopreserved valves [15]. The immunogenicity of valve allografts seems to be mediated by the presence of endothelial cells covering the luminal surface, since these cells form the first barrier between allo-reactive immune system and the valve transplant [16]. On the other hand, the presence and the role of professional antigen presenting dendritic cells in valve allograft rejection remain controversial. In the past, only few histological studies have investigated specific cellular components of valve allografts, which may be fundamental for the valvular immunogenicity [17, 18]. In the study presented in chapter 3, we tried to clarify the cellular basis of the immunogeneicity of both aortic and pulmonary valve allografts. The effect of cryopreservation on the presence and antigen expression of endothelial cells and mononuclear cells including dendritic cells were investigated. The fact that dendritic cells are not profoundly present in any valve allograft specimen may imply reduction of the immunogenicity of human valves when compared to solid organs. Nevertheless, activated endothelial cells were abundantly present in the vasa vasorum of the adventitial layer. Together with an intact luminal endothelial layer, these activated endothelial cells expressing HLA

class II and adhesion molecules may contribute to additional immunogenicity of both fresh and cryopreserved valve allografts. In our series of valve allografts, no significant differences in the distribution of immune stimulatory cells were found between heart beating and non heart beating donors. This result suggests that valve immunogenicity was not influenced by prolonged ischemia (maximal 24 hour). In addition, the process of cryopreservation only reduced the cellularity in the valve matrix but not the endothelial lining of the valve lumen and vasa vasorum. On the other hand, significant differences were found between aortic and pulmonary valve allografts. The numbers of native donor derived T-lymphocytes and macrophages, which have immune stimulatory capacities, are more frequently found in regions with pre-existing lesions and are more often seen among aortic valve allografts. These findings suggest that aortic valve allografts are more immunogeneic than pulmonary valves, and therefore may induce a stronger immune response. This observation could give additional explanation for the better clinical performance of pulmonary valve allografts compared to aortic valve allografts used for RVOT reconstruction. However, our *in vitro* experiments did not show significant difference in T cell activation between aortic or pulmonary valve recipients.

The results of our immuno-histological study give evidence for a cellular basis of allograft immunogenicity. Previous studies have already indicated that clinical implantation of immunogeneic valve allografts could induce both humoral and cellular immune responses against donor specific HLA class I and II molecules [19-21]. However, definitive proof for anti-donor immune reactions causing structural valve allograft deterioration has not been provided yet. The restricting factor is that after valve allograft implantation, histological monitoring of valve tissue for signs of rejection is not feasible in the clinical setting. Histo-pathological studies involving valve allografts, which are explanted during re-operation or autopsy, reported equivocal results [4-8]. Mononuclear infiltrations in the leaflets were observed in relative early explants while late explants showed areas of fibroblast dysplasia combined with calcification resulting in thickening of the leaflets. In most late explants decreased cellularity or even total acellularity of the matrix was seen. The diversity in abnormal valve structure among dysfunctioning explants suggests different pathways leading to structural allograft degeneration. One of the main issues in valve allograft degeneration is the role of specific anti-donor immune response. In practice, very few explants could be examined (except in cases of infective endocarditis) between the 10^{th} postoperative day and 12^{th} month after valve implantation. Therefore, alternative *in vitro* studies have been performed in order to monitor tissue destruction in a non-invasive manner. In the first part of this thesis, three clinical related *ex vivo* studies were conducted to demonstrate indirectly tissue damage by the use of specific *in vitro* techniques derived from organ and tissue transplantation studies (chapter 4-6). After heart or corneal transplantation, the increase of a specific proportion of anti-donor reactive Helper or Cytotoxic T-lymphocytes in peripheral blood was associated with graft rejection. Based on this concept, we monitored in peripheral blood of valve allograft recipients the kinetics of interleukin(IL)-2 producing helper T lymphocytes and

destructive cytotoxic T lymphocytes. Among organ transplant recipients, increase of donor-specific T cell frequency in peripheral blood were only seen during rejection episodes compared to periods of stable graft function. Unlike organ transplant recipients, almost all cryopreserved valve allograft recipients demonstrated increase of donor-specific Helper and Cytotoxic T cell frequencies. The more pronounced increase of circulating T lymphocyte frequencies could be the result of repeated donor-recipient HLA mismatches donor and the lack of immune suppressive therapies. Nevertheless, the frequencies of circulating donor specific Helper and Cytotoxic T-lymphocytes measured after valve allograft implantation were in the same range as found during rejection of cardiac or corneal transplants and therefore may indicate tissue injury [22-24].

Interestingly, the maximum frequency of circulating Helper T cells in valve allograft recipients was seen between 1 to 3 months after implantation, while for the Cytotoxic T cells it was in the period 6 and 12 months. The early increase of Helper T cells in the circulation was conform the time after which circulating anti-donor IgG antibodies became apparent [20]. The switch from IgM to IgG antibodies, which are produced by B lymphocytes, is dependent on interaction with Helper T lymphocytes. Our observation may illustrate the sequence of primary activation of Helper T cells, which can modulate other effector cells, e.g. cytotoxic T and B cells, which subsequently attack and damage the graft. In addition to the cytotoxic properties of circulating anti-donor antibodies, valve allografts are also subjected to destructive actions of cytotoxic T lymphocytes. Earlier analysis of T-lymphocytes, propagated from valve allograft explants, revealed the presence of Cytotoxic T cells with donor-specificity [19]. The observation proclaims active cytotoxic T lymphocytes infiltration in valve allografts. In heart transplantation, cytotoxic T cells were cultured from diagnostic endocardial biopsies during acute rejection. At the same time, an increase of cytotoxic T cells with high avidity for donor HLA class I antigens was seen in peripheral blood [23]. Among valve allograft recipients we found a continuous increase of cytotoxic T cell frequency with high avidity for donor antigens in the first 12 months after implantation, which may indicate immunological damage of valve tissue during the first year. However, this presumed tissue injury did not result in valve dysfunction or valve-related re-operation at that time. Also in cardiac transplantation, histological signs of rejection did not correlate with echocardiographical signs of graft dysfunction [25]. Nevertheless, similar to heart transplantation, early damage of valve tissue may influence the durability of the valve allograft on the long run [26].

Interestingly, as illustrated in figure 1A of chapter 6, some of the valve allograft recipients demonstrate sustained elevation of Cytotoxic T cell frequencies even after the first 12 months. Clinical follow up studies may establish the hypothesis of a maintained cellular immune activation leading to more early or more severe structural deteriortation of valve allografts. An other explanation for high Cytotoxic T cell frequencies for a prolonged period may be associated with an increase of valve viability or cellularity which results in an increased antigenic load.

Chapter 6 also describes that no difference was found in the kinetics of Cytotoxic T cells among pediatric or adult valve allograft recipients, although more pulmonary valve allografts were implanted in the pediatric group. However, clinical follow-up studies have repeatedly outlined better allograft durability in the adult population [27, 28]. We presume that the difference in allograft survival is not only based on the difference in the immunogenicity of the valve types but is associated with the intrinsic structural difference between aortic and pulmonary valves. It has been described that aortic valve allografts contain more calcium and therefore are more prone to calcify [29]. Additionally, in the pediatric population, almost all valve recipients may outgrow the valve allograft, which becomes increasingly stenotic and needs replacement.
It seems that deterioration of human tissue valve is a multi factorial process in which anti-donor immune activation may play an important role in the durability of these valve allografts.

Immunological aspects of valve allograft implantation: Experimental animal studies

Various animal models have been used in the past to investigate the concept of immunologically based valve allograft degeneration. Animal models are often used in transplantation studies because of two important advantages. Firstly, it is possible to have access to the graft at specific intervals after transplantation. Secondly, by the use of inbred strains, the effect of tissue antigen mis-match can be studied in an appropriate allogeneic environment. Especially in rodent models, inbred strains are available with well-defined Major Histo-Compatibility typing. On the other hand, extrapolation of results obtained from animal models to human may be difficult because of major differences in the physiology of important biological systems, including the immune system. Especially among small experimental animals, differences of implantation techniques (orthotopic or heterotopic) may hinder straightforward interpretation of the observations. First *in vivo* experiments were performed primarily to investigate the antigenicity of valvular tissues. Secondly, allogeneic transplantation models (orthotopic and heterotopic) were introduced to study histological signs of valve allograft rejection. Finally, the possible correlation between immune activation against donor antigens and valve allograft dysfunction could be studied by the combination of both *in vivo* and *in vitro* experiments.
In the past, monitoring of donor specific immune activation was assessed by sensitization studies including secondary skin transplants. The time of skin graft rejection is indicative for the degree of sensitization [30]. The major objection of this technique is the analysis of skin rejection, which is easily subjected to differences in individual interpretation. Due to the lack of sensitivity, this method may not be used for monitoring of the immune response after allogeneic valve transplantation. Nowadays, more sensitive and objective *in vitro* techniques are available for analysis of the kinetics of

donor specific humoral and cellular immune responses. Experimental studies involving transplantation of fresh aortic valves between histo-incompatible inbred rat strains revealed the induction of antibodies directed against donor endothelial cells [31]. Additionally, the induction of donor-specific Cytotoxic and Helper T lymphocytes was observed after heterotopic transplantation of fresh aortic valve allografts [32, 33]. Cryopreservation of aortic valves appeared to reduce but not abrogate the allogeneic response.

In the past, large experimental animals (canine, sheeps or calves) were used to study immunologically related degeneration of valve allografts. The use of large animals brought the possibility for orthotopic implantation of valve allografts. However, the use of larger animals from non-inbred strains may include reactions to non-histocompatibility antigens that may lead to increased variability in results. The use of inbred rat strains gives us the possibility to study the effect of Major Histocompatibility mismatch on the induction of an anti-donor immune response and the deterioration of valve structure. Additionally, the effect of variable degree of histo-incompatibility could be determined. However, implantation of heart valves in an orthotopic site remains unfeasible in rodent models. Heterotopic implantation may include non-specific reactions masking changes in valve structure that are specific for histoincompatibility. In addition, heterotopic implantation may alter the physiological valve function and therefore interfere with analysis regarding immune related valve dysfunction. Our heterotopic aortic valve transplantation model in rats managed to solve the problem of size-mismatch between the aortic annulus and the diameter of the abdominal aorta. This implantation technique model minimized non-specific valve deterioration and gave access for functional analysis of transplanted valves. The results of our *in vivo* studies described in chapters 7 to 10 in this thesis demonstrated that implantation of allogeneic valve is followed by a donor-reactive Helper T lymphocyte response. The cellular activation corresponded with histological signs of acute and chronic rejection. Syngeneic transplantation did not cause increase of allo-reactive Helper T lymphocyte frequencies, while syngeneic grafts demonstrated reversible minor inflammation, early after transplantation. Interestingly, massive infiltration of mononuclear cells seen early after allogeneic transplantation was not associated with valve incompetence. Dysfunction of the valve was observed by the time that allograft leaflets had become dysmorphic. This observation demonstrated that the valve function is not impaired during acute rejection of valve allografts. However, acute rejection results in dysmorphic and acellular valve leaflets on the long run, which was associated with allograft dysfunction. This phenomenon may explain our clinical observation in which no valve dysfunction was seen during the first year after valve allograft implantation. Nevertheless, early immune activation may enhance the risk of late allograft dysfunction. Cryopreservation appeared to reduce but not abrogate the immune response as cellular reactivity remained high in the allogeneic recipient. Meanwhile, cryopreservation induced non-immunological aortic valve allograft injury in rats, which clearly effected the valve performance [34]. Nevertheless, these results suggest that cryopreservation of valve allograft reduces the immunogeneic load of the allograft leading to a

decreased but not abrogated anti-donor immune reaction. Matching for histocompatibility complex antigens may result in reduction of the immune response against donor tissue valves, which can contribute to the improvement of valve allograft durability.

Clinical consequences and recommendations for valve allograft implantation

Both fresh and cryopreserved human tissue valves contain cells, including activated endothelial cells, with immune stimulatory abilities. We have to be aware of the fact that implantation of cryopreserved human valve allograft is not immunologically innocent. The induction of donor-specific humoral and cellular immune reaction among most valve allograft recipients may lead to immunologically induced tissue damage. However, the clinical consequence of such immune related damage is still unknown. It has been suggested that multiple factors are underlying the mechanism of tissue valve failure, including donor, recipient and technical factors [2]. Analysis of individual factors affecting allograft durability is complex because of possible interactions between these variables. By elimination of these variables via controlled experimental studies, we were able to demonstrate the importance of immunological responses in the process of valve allograft degeneration associated with valve dysfunction. Interestingly, in our rat studies, early cellular infiltration in the valves was not associated with valve dysfunction. Impairment of valve function was seen in association with structural deformation of the valve later after transplantation. Extrapolation of these results to the clinical setting suggests that acute rejection of the valve graft may not directly be associated with valve dysfunction, while function impairment may appear later. Moreover, clinical manifestation of valve dysfunction may become apparent after a more extended period following transplantation. Therefore the clinical consequence of immune activation is only assessable in association with long-term follow up studies.

Future studies may include extension of *in vitro* tests regarding the numbers of patients. Additionally, the frequency kinetics of circulating immune competent cells can be related to the clinical (echocardiographic) follow up of the patients. In order to study the long-term clinical effect of immuno-histoincompatibility on graft performance, routine HLA typing of valve donor and recipients must be performed. The results of these studies may then identify patients with higher risk for early valve failure due to immunological reactions. Different strategies for reducing the immunological "side-effects" of valve allograft implantation are conceivable. Matching of HLA class I and class II between donors and recipients have proven to be effective for prolonged survival of organ transplants. However, for valve allografts, it may not be feasible because of the increasing shortage of donor valves. Modulation of valve antigenicity may lead to a less aggressive immune response of the acceptor, which probably increases the durability of valve allografts. As the viability of valve allografts may be related to the durability of graft, reducing all interstitial cells of the valve

may increase the risk of early valve degeneration. Selective reduction of cells expressing transplantation antigens may be another reasonable option, provided that we know which cells are responsible for the immunogenicity of the allograft. By phenotypic manipulation of the donor endothelium or interstitial cells in valve allografts, the allo-reactivity could be modulated. This tissue-engineering concept including decellularisation of the allogenic or even xenogenic valves followed by reseeding with recipients' autologous endothelial and fibroblast cells is still under investigation. Promising pre-clinical results have been reported [35-38]. In a study describes by Zund *et al.*, human aortic myofibroblast were seeded on polyglycolic acid (PGA) meches and the effect of different seeding intervals was investigated by direct cell counting or MITT assay [35]. Scanning electron microscopical analysis of the incubated meshes was performed for tissue development. It is possible to develop tissue structures by culturing human myofibroblast on polymeric meshes. The optimal culture interval for human myofibroblast was 24 hours and produced more solid tissue like structure. However structural changes due to functional stress is not covered in this study. Nevertheless, the possibility to use non-biological scafolds reduces the chance of immunological activation. Hoerstrup *et al.* studied the structural changes of tissue engeneering valves in vivo, by implanting trileaflet heart valves made from novel bioabsorbable polymers which are sequentially seeded with autologous myofibroblast and endothelial cells, in the pulmonary circulation of lambs [36]. The constructs were grown for 14 days in a pulse duplicator in vitro system under gradually increasing flow and pressure conditions. The results include normal heart valve microstructure, mechanical properties and extracellular matrix formation up to 5 months. Complete degradation of the polymers occured at 8 weeks. Steinhoff *et al.* demonstrated in a sheepmodel the advantages of in-vitro-cultivated autologous vascular wall cell in an acellular allogeneic pulmonary heart valve scafold [37]. Trypsin/EDTA solution were used to decellularized the valve followed by static reseeding with myofibroblast for 6 days and endothelial cells for 2 days. Unseeded allogeneic acellular valves showed degeneration and no interstitial tissue reconstitution, while engeineered valves showed complete histological reconstitution. Zeltinger and colleagues studied tissue-engineered pulmonary valves by seeding of neonatal fibroblasts onto decellularized, porcine aortic valves [38]. This model includes culture of the acellar xenograft valve scafold in a dynamic pulsatile fluid flow. Viability of the cells were indicated by MTT viability staining. These fibroblast were mitotic and were able to synthesize extracellular matrix proteins, thereby supplementing the existing procine matrix. These studies illutrate the possibilities of creating tissue-engineered heartvalves. Another possible intervention strategy may be immunosuppressive therapy. The introduction of mild immune suppressive therapy during a reduced period of time is conceivable since valve allografts are less immunogenic than solid organs. Data from a study performed in a pediatric population however show no differences in clinical allograft performance in patients with or without azathioprine therapy [39]. Nevertheless, the duration and the choice of the immune suppressive therapy may not be adequate since the increase of circulating donor-specific T cells were found in

the first year after implantation. Moreover, the follow-up period of the patients in this study may not be sufficient, since valve dysfunction may become apparent long after primary tissue injury. Nevertheless, the use of immunosuppressive drugs should be limited to high-risk patients since the negative side effects of immunosuppression may not outweigh its advantages. Still, implantation of valve allograft could induce a donor-specific immune response, which results in active sensitization of valve recipients. Sensitized patients may develop a more severe immune response when challenged by similar tissue antigens during successive valve allograft implantation at re-operations. This may have negative effect on the durability of each consecutive valve allograft. More importantly, patients sensitized for HLA molecules may have difficulties in finding suitable organ transplants in future, due to increased possibility of positive cross-matches.

Conclusions

1. Heart valve allografts used in right ventricular outflow tract reconstruction have satisfactory mid-term results but they also are subjected to longterm structural degeneration.

2. Fresh and cryopreserved valve allografts contain activated endothelial cells and mononuclear cells expressing Human Leukocyte Antigens (HLA) class II molecules.

3. Implantation of cryopreserved valve allografts is followed by an increase of donor-specific helper T lymphocyte and cytotoxic T lymphocyte frequencies among peripheral blood mononuclear cells.

4. The increase of circulating cytotoxic T lymphocyte frequencies and their avidity for donor Human Leucocyte Antigen (HLA) class I molecules are comparable in pediatric and adult valve allograft recipients.

5. Fresh aortic valves transplanted across a Major Histo Compatibility (MHC) barrier induce a donor reactive helper T cell response, which is associated with cellular rejection and dysfunction of the valve allograft in a rodent model.

6. Immunogeneicity of cardiac valve allografts is reduced but not abrogated by cryopreservation of the graft.

7. The process of crypreservation induced non-immunogical degeneration of aortic valve grafts in rats.

References

1. Cohn LH, Lipson W. Selection and complications of cardiac valvular prostheses. In: Baue AE, Geha AS, Hammond GL, Laks H, Nauheim KS, editors. Glenn's Thoracic and Cardiovascular Surgery. Vol 2. 6th ed. Stamford (CT): Appleton & Lange;1996: 2043-2055
2. McGiffin DC. Invited letter concerning: Leaflet viability and the durability of the allograft aortic valve. J Thorac cardiovasc Surg 1994;108:988-90
3. Baskett RJ, Ross DB, Nanton MA et al. Factors in the early failure of cryopreserved homograft pulmonary valves in children: preserved immunogenicity ? J Thorac cardiovasc Surg 1996;112:1170-9
4. Rajani B, Mee RB, Ratliff NB. Evidence for rejection of homograft cardiac valves in infants. J Thorac Cardiovasc Surg 1998;115:111-7
5. Vogt PR, Stallmach T, Niederhauser U et al. Explanted cryopreserved allografts: a morphological and immunohistochemical comparison between arterial allografts and allograft heart valves from infants and adults. Eur J Card-Thorac Surg 1999;15:639-645
6. Neves J, Monteiro C, Santos R et al. Histologic and genetic assessment of explanted allograft valves. Ann Thorac Surg 1995;60:S141-5
7. Mitchell RN, Jonas RA, Schoen FJ. Pathology of explanted cryopreserved allograft heart valves: comparison with aortic valves from orthotopic heart transplants. J Thorac Cardiovasc Surg 1998;115:118-27
8. Goffin YAH, Henriques de Gouveia R, Szombathelyi T et al. Morphologic study og homograft valves before and after cryopreservation and after short-term implantation in patients. Cardiovasc Pathol 1997;6:35-42
9. Balch CM, Karp RB. Blood group compatibility and aortic valve allotransplantation in man. J Thorac Cardiovasc Surg 1975;70:256-259.
10. Yacoub MH. Applications and limitations of histocompatibility in clinical cardiac valve allograft surgery. In: Yankah AC, Hetxer R, Miller DC, Ross DN, Sommerville J, Yacoub MH eds. Cardiac valve allograft 1962-87. New York, Springer-verlag, 1988:95-102
11. Kadner A, Chen RH, Mitchell RN et al. Lack of ABH-antigen expression on human cardiac valves. J Heart Valve Dis 2000;9:512-516
12. Wood K, editor. The handbook of transplant immunology. MedSci publications 1995
13. Dignan R, O'Brien M. Hogan P et al. Influence of HLA Matching and associated factors on aortic valve homograft function. J Heart Valve Dis 2000;9:504-511
14. Smith JD, Hornick PI, Rasmi N et al. Effect of HLA mismatching and antibody status on "homovital" aortic valve homograft performance. Ann Thorac Surg 1998;66:S212-5
15. Gall KL, Smith SE, Willmette CA, O'Brien MF. Allograft heart valve viability and valve-processing variables. Ann Thorac Surg 1998;65:1032-8
16. Simon A, Wilhelmi M, Steinhoff G et al. Cardiac valve endothelial cells: relevance in the long-term function of biologic valve prostheses J Thorac Cardiovasc Surg 1998;116:609-16
17. Yacoub M, Suitters A, Khaghani A et al. Localization of Major Histocompatibility Complex (HLA, ABC and DR) antigens in aortic homografts. In: Bodnar E, Yacoub M eds: Biologic and bioprosthetic valves. Proceedings of the third international symposium. New York, York medical Books 1986:65-72
18. Strutton G, Hogan P, Green M et al. Immunohistochemical analysis of dendritic cells in human aortic valve allografts Abstract nr.40 World symp. on heart valve disease 1999:92

19. Hoekstra, FM, Knoop CJ, Vaessen LM et al. Donor-specific cellular immune response against human cardiac valve allografts. J Thorac Cardiovasc Surg 1996;112:281-6
20. Hoekstra FM, Witvliet M, Knoop CJ et al. Donor-specfic anti-human leukocyte antigen class I antibodies after implantation of cardiac valve allografts. J Heart Lung Transplant 1997;16:570-2
21. Hoekstra FM, Witvliet M, Knoop CJ et al. Immunogeneic human leukocyte antigen class II antigens on human cardiac valves induce specific alloantibodies. Ann Thorac Surg 1998;66:2022-6
22. Vaessen LMB, Daane CR, Maat APWM et al. T helper frequencies in peripheral blood reflect donor-directed reactivity in the graft after clinical heart transplantation. Clin Exp Immunol 1999;118:473-9
23. Van Emmerik NE, Vaessen LM, Knoop CJ et al. Kinetics of circulating cytotoxic T lymphocyte precursors that have a high avidity for donor antigens: correlation with the rejection status of the human cardiac allograft. Transpl immunol 1998;6:153-60
24. Roelen DL, van Beelen E, van Bree SP et al. The presence of activated donor HLA class I reactive T lymphocytes (CTLs) is associated with rejection of corneal grafts. Transplantation 1995;59:1039-42
25. Valentine HA. Rejection surveillance by doppler echocardiography. J Heart Lung Transplant 1993;12:422-6
26. Hauptman PJ, Nakagawa T, Tanaka H, Libby P. Acute rejection:Culprit or Coincidence in the pathogenesis of cardiac graft vascular disease? J Heart Lung Transplant 1995;14:S173-80
27. Niwaya K, Knott-Craig CJ, Lane MM et al. Cryopreserved homograft valves in the pulmonary position: risk analysis for intermediate-term failure. J Thorac Cardiovasc Surg 1999;117:141-7
28. O'Brien MF. The 28-year, 99.3% follow up of 1022 homograft aortic valve replacement patients. J Heart Valve Dis 2001;10:334-345
29. Livi U, Abulla AK, Parker R et al. Viability and morphology of aortic and pulmonary homografts. J Thorac Cardiovasc Surg 1987;93:755-60
30. Thiede A, Timm C, Bernhard A et al. Studies on the antigenicity of vital allogeneic valve leaflet transplants in immunologically controlled strain combinations. Transplantation 1978;26:391-95
31. Yankah AC, Wottge HU, Dreyer W et al. Kinetics of endothelial cells of aortic valve allografts transplantaed heterotopically in inbred rats. In: Bodnar E, Yacoub MH, eds. Biologic and bioprosthetic valves. New York: Yorke Medical Books, 1986:73-84.
32. Zhao X, Green M, Frazer IH et al. Donor-specific immune response after aortic valve allografting in the rat. Ann Thorac Surg 1994;57:1158-63
33. Oei FB, Stegmann AP, Vaessen LM et al. Immunological aspects of fresh and cryopreserved aortic valve transplantation in rats. Ann Thorac Surg 2001;71:S379-84
34. Legare JF, Lee TD, Ross DB. Cryopreservation of rat aortic valves results in increased structural failure. Circulation 2000;102:III75-78
35. Zund G, Ye Q, Hoerstrup SP, Schoeberlein A et al. Tissue engineering in cardiovascular surgery: MTT, a rapid and reliable quantitative method to asses the optimal human cell seeding on polymeric meshes. Eur J Cardiothorac Surg. 1999;15:519-24.
36. Hoerstrup SP, Sodian R, Daebritz S. et al. Functional living trileaflet heart valves grown in vitro. Circulation. 2000;102 (19 Suppl 3):III44-9.
37. Steinhoff G, Stock U, Karim N et al. Tissue engineering of pulmonary heart valves on allogeneic acellular matrix conduits: in vivo restoration of valve tissue. Circulation 2000;102(19 Suppl 3):III50-5
38. Zeltinger J, Landeen LK, Alexander HG et al. Development and characterizartion of tissue-engineered aortic valves. Tissue Eng 2001;7(1):9-22
39. Shaddy RE, Lambert LM, Fuller TC et al. Prospective randomized trial of azathioprine in cryopreserved valve allografts in children. Ann Thorac Surg 2001;71:43-7

CHAPTER 12

Summary & Samenvatting

F.B.S. Oei

Summary

In respect of the hemodynamical properties, donor valve allografts have proven to be the perfect biological prostheses for surgical correction of congenital or acquired heart valve diseases. Human valve allografts are subjected to progressive structural degeneration, leading to a limited durability. Different interrelated factors have been suggested to cause tissue degeneration. An immunological cause of tissue degeneration is feasible since valve allografts are implanted without matching of tissue antigens. The immunogenicity of either fresh or cryopreserved valve allograft tissue has been indicated by several *ex vivo* studies demonstrating the induction of specific humoral and cellular immune responses after allogeneic valve implantation. Nevertheless, a direct association of anti-donor immune response and valve tissue deterioration is not yet found. This thesis includes clinical *ex vivo* and experimental *in vivo* studies for analysis of donor-specific immune reactivity, which may affect the structural integrity and the function of allogeneic valve grafts. The role of Helper T and Cytotoxic T lymphocytes were highlighted, as these cells are regarded as the important cells in allograft rejection.

Chapter 1: General introduction. The first chapter describes the history of human donor valve allografts in cardiac surgery, followed by a brief summation of recent reports regarding the clinical outcome of valve allograft implantation. The majority of human donor valves demonstrated structural deterioration on the long run. Since implantation of allografts is performed without considering the immunogenicity of the allogeneic valve, rejection of the graft may occur. Immune activation may play an important role in clinical allograft degeneration. To improve our discernment regarding the concept of allogeneic tissue rejection, the basic principles of transplant immunology are discussed. The current knowledge concerning the immunogenicity of valve allografts is further reviewed by referring to previous animal and human studies. Finally, the aims of the studies incorporated in the thesis are outlined.

PART I: PATIENT RELATED STUDIES

Chapter 2: Right ventricular outflow tract reconstruction with an allograft conduit. This chapter describes a retrospective study regarding the clinical outcome of valve allografts used in right ventricular outflow tract (RVOT) reconstruction. To identify risk factors for early valve dysfunction, single and multivariate analysis were performed for presumed risk factors. A series of 316 allografts (246 pulmonary, 70 aortic) were implanted in 297 patients for reconstruction of the RVOT. Kaplan-Meier analysis was used for survival, valve-related re-operation and events, while Cox regression analysis was used for evaluation of potential risk factors. The mean age at operation was 18 years (7 days-61 years) and the mean follow-up was 4 years (2 days-12 years). Patient survival was 90% at 5

years and 88% at 8 years. Twenty-four re-operations were required for allograft dysfunction in 23 patients while 21 allografts were replaced. The 5 and 8 years freedom from valve-related re-operation was 91% and 87% respectively. Twenty-nine valve-related events were reported. Freedom from valve-related events was 90% at 5 years and 84% at 8 years. Risk factors for accelerated allograft failure were extra-anatomical position of the allograft ($p=0.03$) and the use of an aortic allograft ($p=0.02$). We conclude that RVOT reconstruction with an allograft conduit has good mid-term results, however, progressive allograft degeneration is noted. In the absence of data regarding tissue HLA mismatches, the extra-anatomical position of the allograft and aortic allografts appeared to be the only risk factors for early allograft failure.

Chapter 3: The presence of immune stimulatory cells in fresh and cryopreserved valve allografts.
In this immuno-histochemical study, we analyzed the presence and distribution of activated endothelial and mononuclear cells, expressing adhesion and HLA class II molecules in 16 aortic and 13 pulmonary heart valve allografts (HVA) obtained from 12 heartbeating and 9 non-heartbeating tissue donors. The effect of cryopreservation regarding the presence of immune stimulatory cells was analyzed by symmetric dissection of the grafts. Activated endothelial cells, expressing adhesion molecules (PECAM-1, VCAM-1) and HLA class II molecules covered at least 50% of the luminal surfaces. A comprehensive vascular network was found in the adventitial layer of the vascular wall, covered entirely by activated endothelial cells. HLA class II positive macrophages (CD68) and T-lymphocytes (CD3) were found scattered in the stroma and subendothelial layer of the valve leaflets. Dendritic cells expressing both S100 and CD45 were not found in immuno-double stained sections. Cryopreserved HVA demonstrates increased numbers of pycnotic cells and reduced expression of adhesion molecules of luminal endothelial cells but not vaso vasorum endothelial cells. Although immunohistochemical staining can not evaluate the viability of the cells, we believe that viable and activated donor cells expressing allogeneic molecules are present at transplantation. In an allogeneic setting, immune stimulatory cells may induce a destructive immune response. The absence of dendritic cells is compensated by the preservation of a comprehensive network of activated endothelial cells in the outer layer of the allograft wall, which suggest induction of allo-response by indirect antigen-presentation.

Chapter 4: Increase of helper T lymphocytes after transplantation of cryopreserved human valve allografts. A specific immunological response against immunogeneic valve grafts could be involved in the process of allograft degeneration. Helper T lymphocytes play an important intermediate role in cellular and humoral immune response leading to allograft rejection. The increase of donor-specific helper T lymphocyte precursor (HTLp) frequencies in peripheral blood is correlated with organ rejection, therefore it may serve indirectly as evidence for tissue rejection. HTLp frequency was determined in sequentially taken peripheral blood of 13 valve allograft recipients, prior to and after valve

implantation. Enumeration of HTLp was performed by the use of limiting dilution assays and interleukin-2 bioassays. In pre-transplant blood samples, each patient demonstrated their individual baseline HTLp frequencies. After implantation of cryopreserved valve allografts, anti-donor HTLp frequencies significantly increased in 11 of the 13 patients ($p=0.02$). The maximal frequencies of donor-specific HTLp were found 1-2 months after valve implantation. The interval is comparable to the time after which circulating donor-specific HLA class I and II antibodies became detectable (Hoekstra *et al.*). The results of this study indicate that implantation of valve allografts induces frequency increase of donor-specific HTLp in peripheral blood of the recipients. Analogue to organ transplantation, HTLp may play a crucial role in events that lead to valve damage.

Chapter 5: Induction of cytotoxic T lymphocytes with destructive potential after cardiac valve allograft implantation. Cytotoxic T lymphocytes (CTL) are presumed to be the major effector cells causing graft destruction. Previous studies concerning heart and corneal transplantation demonstrated a strong association between the frequency increase of cytotoxic T lymphocytes (CTL) with high avidity for donor antigens in peripheral blood and graft rejection. The kinetics of donor-specific CTL precursors (CTLp) and their avidity for donor antigens was monitored in peripheral blood of valve allograft recipients, as indirect indication for tissue injury. Enumeration of CTLp was performed by using a Limiting Dilution Assay (LDA) in sequentially taken peripheral blood samples of 15 patients, obtained during the first 12 months after valve implantation. The avidity of CTLp was determined by the use of CD8 monoclonal antibodies during the cytotoxic phase of the assay. In 14 out of 15 patients (93%) a significantly increase of total donor-specific CTLp was seen at 6 months ($p=0.045$) after valve implantation and remained significant increased up to 12 months ($p=0.015$). Additionally, a more significant increase was observed in the fraction of circulating CTLp with high avidity for donor antigens already within the first 3 months after implantation ($p=0.026$). This study illustrated the immunogenicity of cryopreserved valve allografts by demonstrating the ability of these valves to induce a donor-specific CTLp response which may have destructive potentials.

Chapter 6: Circulating donor-specific cytotoxic T lymphocytes with high avidity for donor HLA in pediatric and adult cardiac valved allograft recipients. Several clinical studies have reported young recipient age as a significant risk factor for early valve allograft failure. A more effective immune system was suggested to cause this difference. This theory was studied by comparing the kinetics of CTLp frequencies and avidity for donor HLA in 9 adult and 6 pediatric valve recipients. Peripheral blood samples were obtained up to 3 years after valve implantation. Enumeration and avidity testing of CTLp was conform the methods described in chapter 5. The total frequency of donor-specific CTLp increased significantly in the period 6 to 12 months after valve implantation. The percentage of high avidity CTLp showed an increase during the first 6 months after valve implantation and remained increased even after 12 months. No significant differences in the kinetics of CTLp frequencies or avidity were

found between pediatric and adult valve recipients. The difference in clinical outcome may be caused by non-immunological factors such as sizing, surgical distortion of the valve graft or simply outgrow of the valve allograft.

PART II: EXPERIMENTAL ANIMAL STUDIES

Chapter 7: A size-matching heterotopic aortic valve implantation model in the rat. This chapter described a size matching *in vivo* model for analysis of immune mediated cardiac valve degeneration. Structural degeneration of cardiac valve allografts may be related to technical factors like size-mismatch or immunological factors, both resulting in mononuclear cell infiltration, thrombosis, intimal proliferation and fibrosis of the valve graft. To exclude non-immunological confounding factors, a proper transplantation model must be provided. Inbred rat strains are frequently used in transplantation studies because of their defined major histo-compatibility (MHC) antigens. However, orthotopic transplantation of valve grafts in these small animals is not feasible. Existing heterotopic transplantation models include end-to-end anastomosis of donor aortic valve graft in the abdominal aorta of the recipient. Size differences between the aortic valve annulus and the abdominal aorta may cause non-immunological tissue deterioration. End-to-side anastomosis may provide a technical adaptation for the size-mismatch. 100% survival and patency of the aortic grafts at 21-day was observed. Valve function, assessed by retrograde injection, revealed valve incompetence among the allogeneic valves. No fibrosis or intimal thickening in the syngeneic valves was found. These results indicate that non-immunological changes are minimized in this model. Additionally, functional analysis of the valve grafts is feasible. This heterotopic implantation technique enables us to study the association between allogeneic responses and allograft degeneration in a technically unbiased manner.

Chapter 8: Heart valve dysfunctioning due to cellular rejection in a novel heteroptic transplantation rat model. The importance of immune related tissue deterioration in the process of valve dysfunction was studied in a heterotopic aortic valve transplantation model. In a series of 10 allogeneic (WAG-BN or WAG-DA) and 10 syngeneic (WAG-WAG or DA-DA) freshly transplanted aortic valve grafts, the function and (immuno-)histology of the grafts was examined after 21 days. 80% of the allogeneic transplants were incompetent while among syngeneic grafts it was 10%. Allogeneic valve leaflets appeared deformed and non-cellular with extensive infiltration of $CD4^+$, $CD8^+$ T-lymphocytes and $CD68^+$ macrophages in the adventitia and media layer. Syngeneic valve grafts had normal valves structures without fibrosis or intimal thickening, while minimal T-lymphocytes and macrophages were found in the necrotic myocardial rim and adventitial layer. This study demonstrated that transplantation of fresh aortic valves between MHC-incompatible rat-strains resulted in allograft rejection which was associated with valve incompetence.

Chapter 9: Frequencies of donor reactive helper T lymphocytes correlate with rejection of fresh allogeneic aortic valve grafts in rats. The study described in chapter 8 demonstrated that transplantation of allogeneic valve grafts results in cellular rejection of the valves. After valve allograft transplantation, circulating Helper T-lymphocyte precursor frequencies (HTLpf) appeared to be increased in the majority of the recipients (chapter 4). However, direct correlation between HTLpf and valve rejection is never made since histological diagnosis of valve rejection can not be made in the clinical setting. In this study the value of HTLpf analysis as indicator for graft rejection was investigated. HTLpf were determined among peripheral blood mononuclear cells and spleen cells at 7 and 21 days after allogeneic (WAG→DA) transplantation of fresh aortic valves in rats. Syngeneic (DA→DA) valve transplantations and allogeneic skin transplantations served as negative and positive control experiments respectively. After 7 and 21 days, the valves were explanted and tested for competence followed by histological analysis. Donor-reactive HTLpf among spleen cells was significantly elevated at the 7th day after transplantation, while the increase in peripheral blood was seen later at the 21st day. Valve allograft competence declined from 71% at day 7 to 0% at day 21 ($p=0.01$). Meanwhile all syngeneic valves remained competent. Mononuclear cell infiltration in the allogeneic leaflets and vascular wall was observed at day 7. At day 21 allograft leaflets appeared acellular and deformed. Syngeneic valve grafts demonstrated mild cellular infiltration in the vascular wall probably as a response to surgery. Fresh aortic valves allografts induce donor-reactive HTLpf, which correlates with early histological rejection and late valve dysfunction. Monitoring of donor-reactive HTLpf in valve allograft recipients may serve as alternative indicator for graft rejection, which ultimately may lead to valve dysfunction .

Chapter 10: Immunological aspects of fresh and cryopresereud aortic valve transplantation in rats. Since the immunogenicity of the allograft is related to the expression of allo-antigens, cryopreservation may affect the immunogenicity of valve allografts. In this study, we investigated the effect of cryopreservation on valve performance, histo-morphology and tissue immunogenicity. Fresh (FA) or cryopreserved (CA) allogeneic and fresh (FS) or cryopreserved syngeneic (CS) aortic valve transplantations were performed. The competence and immuno-histology of the valve grafts were examined after 7 or 21 days after transplantation. Determination of the allo-response was assessed by concanavalin A cell stimulation and analysis of donor-reactive HTLf among peripheral blood mononuclear cells and spleen cells. Fresh and cryopreserved allografts demonstrated progressive loss of competence and infiltration of mononuclear cells while all fresh syngeneic grafts remained competent without signs of rejection. Cryopreservation led to early incompetence in both allogeneic and syngeneic grafts due to the formation of retrovalvular thrombi. The reactivity of T-lymphocytes and the HTL frequencies among both peripheral blood lymphocytes and spleen cells were significantly higher in all allograft (FA+CA) recipients compared to syngeneic recipients.

Cryopreserved allografts elicited a lower allo-response compared to fresh grafts. This study confirmed the immunogenicity of aortic valve allografts. The induction of an anti-donor cellular response is related to histological signs of rejection and the loss of structural integrity leading to allograft dysfunction. Cryopreservation appeared to diminish but not to eliminate the immunogenicity of the allograft. Early formation of retrovalvular thrombi in the cryopreserved valves may be caused by chemical or mechanical factors leading to more thrombogenic grafts. When extrapolating these findings to clinical practice, we may suggest the following sequences; implantation of cryopreserved valve allografts may lead to T-lymphocyte activation, which causes "early" tissue injury and slow degeneration process, finally this may result in "late" allograft dysfunction.

Chapter 11: General discussion. In this chapter, a comprehensive discussion in which the most important results and conclusions of the studies in chapters 2 to 10 are reviewed as related to the aims of the thesis. Possible clinical implications of the conclusions provided by this thesis were discussed together with recommendations for further research. The importance of tissue antigen matching in preventing valve allograft degeneration is difficult to investigate clinically because of the heterogenicity of the HLA antigens. Moreover, the applicability of antigen matching is limited because of the shortage of donor valves. In order to prevent or minimize the adverse effect of immune reaction due to antigen mismatch we suggest that modulation of valve immunogenicity should be the main subject of future research. The identification of "high-risk" patients should also be involved in clinical studies because additional immunosuppressive therapies may prevent accelerated valve deterioration. Prospective randomize trials including newer immunosuppressive drugs with limited side effects and administered within a specific time frame may prolong the durability of valve allografts without the major complications of long-term high-dose immunosuppressive therapies.

Samenvatting

Menselijke donorhartkleppen hebben uitstekende hemodynamische eigenschappen en worden gebruikt bij klepvervangende operaties voor zowel congenitale als verworven hartziekten. Desondanks faalt een groot aantal donorhartkleppen door weefsel-degeneratie. Verschillende factoren kunnen hieraan ten grondslag liggen. Behalve chirurgisch-technische factoren en weefsel eigenschappen kan een immunologische afweerreactie tegen het lichaamsvreemde donorweefsel een belangrijke rol spelen in het proces van weefsel-degeneratie. Menselijke donorhartkleppen worden beschouwd als implantaten met een lage immunogeniciteit (de capaciteit om een immuunreactie op te wekken) en worden geïmplanteerd zonder te "matchen" voor weefsel-antigenen zoals AB0-bloedgroepen of de "Human Leukocyte Antigen" (HLA). Tevens worden de ontvangers van deze donorhartkleppen, niet zoals bij orgaantransplantatie, behandeld met immunosuppressieve medicijnen. Diverse *ex vivo* studies hebben aangetoond dat verse en ingevroren (gecryopreserveerde) donorhartkleppen een donorspecifieke humorale en cellulaire immuunreactie bij klepontvangers opwekken. Hoewel de immunogeniciteit hiermee is aangetoond blijft het onduidelijk of de donorspecifieke immuun-activatie ook leidt tot structurele degeneratie van de donorklep. In dit proefschrift worden met behulp van klinische *ex vivo* en experimentele *in vivo* modellen de immunologische aspecten van allogene klepimplantatie geanalyseerd. De frequentie van Helper en Cytotoxische T lymfocyten in donorklepontvangers is onderzocht, omdat deze cellen een belangrijke, reeds aangetoonde, rol spelen in de afstoting van donororganen. Tevens is het effect van immuun-activatie op weefselintegriteit en klepfunctie onderzocht.

Hoofdstuk 1: Introductie. In dit eerste hoofdstuk van dit proefschrift volgt een korte beschrijving van de geschiedenis van de menselijke donorhartklep. Tevens wordt een overzicht gegeven van de klinische studies naar de resultaten van donorhartklepimplantaties. Uit dit overzicht blijkt dat een groot deel van deze kleptransplantaten op den duur degenereert, ondanks goede klinische resultaten. Omdat bij transplantatie van donorhartkleppen geen rekening wordt gehouden met de immunogeneiciteit van het transplantaat, is een immunologische oorzaak voor implantaat degeneratie niet ondenkbaar. Een beknopte beschrijving van het principe van de transplantatie immunologie volgt, in combinatie met een overzicht van eerdere studies naar de immunologische aspecten van hartklepimplantaties. Tenslotte worden de doelen van de studies in dit proefschrift uiteengezet.

DEEL I: PATIENT GERELATEERDE STUDIES

Hoofdstuk 2: Reconstructie van het rechter ventrikel uitstroomtraject met een allograft conduit. In dit hoofdstuk werd het resultaat beschreven van een retrospectieve studie naar de klinische uitkomsten van reconstructies van het rechter ventrikel uitstroomtraject met donorhartkleppen. Risicofactoren voor een vroegtijdige klepdysfunctie werden met behulp van uni- en multivariate analyse geïdentificeerd. Er werden 316 allograft-implantaties (246 pulmonale, 70 aortale) in 297 patiënten bestudeerd. De Kaplan-Meier-analyse werd toegepast voor evaluatie van de patiëntoverleving, klepgerelateerde re-operaties en morbiditeit, Tevens hebben we gebruik gemaakt van Cox-regressieanalyse voor identificatie van potentiële risicofactoren voor een vroegtijdig klepfalen. De gemiddelde leeftijd van de patienten was 18 jaar (7 dagen –61 jaar). De gemiddelde duur van de follow-up was 4 jaar (2 dagen –12 jaar). De 5- en 8-jaars overleving was respectievelijk 90% en 88%. In totaal werden 24 re-operaties uitgevoerd op basis van klepdysfunctie in 23 patienten. Hierbij werden 21 implantaten vervangen. De 5- en 8-jaars vrijheid voor klepgerelateerde re-operaties was respectievelijk 91% en 87%. Klepgerelateerde morbiditeit kwam 29 maal voor. De 5- en 8-jaars vrijheid van klepgerelateerde morbiditeit was respectivelijk 90% en 84%. Extra-anatomische positie van het implantaat ($p=0.03$) en het gebruik van aortale implantaten ($p=0.02$) waren belangrijke risicofactoren voor vroegtijdig klepfalen. Reconstructie van het rechter ventrikel uitstroomtraject met een menselijke donorhartklep geeft een goed resultaat op de middellange termijn, hoewel progressieve degeneratie van het klepweefsel is geconstateerd. Extra-anatomische posities en het gebruik van aortale kleppen waren de enige risicofactoren voor vervroegd klepfalen. Gegevens omtrent de "mismatch" van transplantatie-antigenen tussen klepdonoren en ontvangers waren onbekend en zijn buiten beschouwing gelaten.

Hoofdstuk 3: De aanwezigheid van immuun-stimulatoire cellen in verse en gecryopreserveerde donor-hartkleppen. In deze immuunhistologische studie werden de aanwezigheid en lokalisatie van geactiveerde endotheel en mononuclaire cellen met expressie adhesie en HLA klasse II moleculen geanalyseerd. 16 aortale en 13 pulmonale klepimplantaten afkomstig van 12 "heartbeating" en 9 "non-heartbeating" donoren werden onderzocht. Door de kleppen in twee symmetrische stukken te verdelen voor een directe analyse of analyse na cryopreservatie, kon het effect van cryopreservatie worden bestudeerd. Geactiveerde endotheelcellen, positief voor adhesiemoleculen PECAM-1 en VCAM-1 en HLA klasse II moleculen, bedekten de klepoppervlakte minimaal voor 50%. In de adventitialaag van de vaatwand, werd een uitgebreid vasculair netwerk gevonden dat geheel bedekt was met geactiveerde endotheel cellen. In de matrix van de klepbladen en de subendotheliale laag van de vaatwand werden geactiveerde macrofagen en T-lymfocyten gevonden. Dendritische cellen werden niet gevonden. Gecryopreseveerde klephelften bevatten meer pycnotische cellen in de matrix, terwijl de luminale

endotheel cellen minder adhesie-moleculen tot expressie brachten. Dat geldt niet voor endotheel cellen in de vasa vasorum.
Implantatie van donorhartkleppen kan een anti-donor immuunreactie induceren, door de aanwezigheid van immuun stimulatoire cellen. Gezien de afwezigheid van dendritische cellen verloopt de activatie van het immuunsysteem waarschijnlijk via indirecte antigeen presentatie.

Hoofdstuk 4: Toename van helper T lymphocyten na implantatie van gecryopreserveerde humane donor-hartkleppen. Specifieke immuunactivatie speelt mogelijk een rol in het proces van donorklep-degeneratie. Helper T lymfocyten zijn belangrijk in cellulaire en humorale afweerreacties die leiden tot afstoting van allogeen weefsel. Uit eerdere studies is bekend dat afstoting van orgaantransplantaten gepaard gaat met een toename van de donor-specifieke helper T lymfocyten precursor frequenties (HTLp) in het bloed. De kinetiek van HTLp frequenties kan worden gebruikt als indirecte graadmeter voor afstoting van het transplantaat. De HTLp frequentie is bepaald in bloed van 13 donorklep ontvangers. Dit is afgenomen op verschillende tijdstippen voor en na implantatie. HTLp frequentie is met behulp van een limiting dilution assay en interleukine-2 bio-assay bepaald. Voor de implantatie werden verschillen gezien in de HTLp frequentie tussen de patiënten. Na implantatie werd een toename van donor-specifieke HTLp frequenties gezien in 11 van de 13 patiënten *($p=0.02$)*. De piek in deze frequenties was zichtbaar 1 tot 2 maanden na implantatie. Dit kwam overeen met het tijdstip waarop donor-specifieke anti-HLA class I en II antilichamen worden gevonden in het bloed van de ontvangers. (Hoekstra *et al.*). Deze studie bewijst dat implantatie van allogene donorhartkleppen leidt tot een toename in donor-specifieke HTLp frequenties bij de ontvanger. Overeenkomstig met orgaantransplantaties, kan dit fenomeen een aanwijzing zijn voor klepbeschadiging.

Hoofdstuk 5: Inductie van destructieve cytotoxische T lymfocyten na donor-hartklepimplantatie. Cytotoxische T lymfocyten (CTL) worden beschouwd als belangrijke effector cellen die schade veroorzaken in het afstotingsproces van het transplantaat. Er is bij hart- en hoornvliestransplantaties een sterke correlatie gevonden tussen toename van de frequentie van CTL met hoge aviditeit voor donor HLA antigenen in het bloed en afstoting van het transplantaat. De kinetiek van donor-specifieke CTL precursors en hun aviditeit voor donor HLA-antigenen hebben we bestudeerd in 15 donorhartklep ontvangers. In de periode tot een jaar na klepimplantatie is bij 15 ontvangers op verschillende momenten bloed afgenomen. Er werd een CTLp frequentie bepaling uitgevoerd middels limiting dilution assay en de aviditeit bepaald met CD8 monoclonale antilichamen tijdens de cytotoxische fase van de assay. 14 van de 15 patiënten (93%) lieten een significante toename zien in de CTLp frequentie 6 maanden na implantatie *($p=0.045$)*. Deze bleef verhoogd op 12 maanden na implantatie *($p=0.015$)*. Daarnaast werd al na 3 maanden een significantere toename gezien in het percentage van CTLp met hoge aviditeit voor donor HLA-antigenen *($p=0.026$)*. Deze studie illustreert enerzijds de

immunogeneiciteit van cryopreserveerde donorhartkleppen en anderzijds de inductie van een destructieve CTLp respons.

Hoofdstuk 6: Circulerende donor-specifieke cytotoxische T lymfocyten met hoge aviditeit voor donor HLA in pediatrische en volwassen donorhartklepontvangers. Klinische follow-up studies hebben aangetoond dat de leeftijd van jonge ontvangers een belangrijke risicofactor is voor vroegtijdig falen van donor hartkleppen. Een meer effectief immuunsysteem is gesuggereerd als de oorzaak voor het vroegtijdig falen in jonge ontvangers. Deze theorie is bestudeerd door de kinetiek van de CTLp frequenties en de CTLp aviditeit van 6 pediatrische en 9 volwassen klepontvangers met elkaar te vergelijken. Bloedmonsters zijn verzameld in de periode tot 3 jaar na implantatie. Analyses van frequenties en aviditeit zijn uitgevoerd met dezelfde technieken en op dezelfde wijze zoals beschreven in hoofdstuk 5. De totale donor-specifieke CTLp frequentie was significant verhoogd, 6 tot 12 maanden na de implantatie. Het percentage CTLp met hoge aviditeit voor donor HLA was verhoogd binnen de eerste 6 maanden na implantatie en zelfs na 12 maanden was een verhoging aantoonbaar. Er werd geen verschil gevonden in de aviditeit en de kinetiek van de CTLp frequentie tussen de pediatrische en volwassen ontvangers. Het verschil in klinische resultaten kan worden verklaard door niet-immunologische factoren zoals de maatvoering en chirurgische beschadiging van het implantaat of het feit dat de ontvanger de donorklep "ontgroeit".

DEEL II: EXPERIMENTELE PROEFDIER STUDIES

Hoofdstuk 7: Een passend heterotoop aortaklep implantatiemodel in de rat. In dit hoofdstuk volgt een beschrijving van een *in vivo* transplantatiemodel om immuun gemedieerde allogene hartklep-degeneratie te kunnen bestuderen. Structurele degeneratie van donorhartkleppen zou kunnen worden veroorzaakt door technische factoren zoals maatvoering en chirurgische beschadiging van het implantaat, of immunologische factoren. Beide veroorzaken mononucleaire celinfiltraten, trombosevorming, intimaproliferatie en fibrose-vorming in het implantaat. Om het effect van donor-specifieke immuunactivatie op de klepdegeneratie te kunnen bestuderen in een *in vivo* model moeten alle niet-immunologische factoren worden uitgesloten. Een passend transplantatiemodel is daarvoor van uiterst belang. Ingeteelde rattenstammen worden frequent gebruikt in transplantatie studies omdat transplantatie antigenen (Major Histo-Compatibility) in deze stammen goed gedefinieerd en stam-specifiek zijn. Helaas is orthotope transplantatie van hartkleppen in ratten niet mogelijk. Bestaande heterotope transplantatiemodellen maken gebruik van end-to-end anastomosen tussen het aorta-transplanaat en de abdominale aorta van de ontvanger. Het natuurlijke verschil in diameter tussen de aortaklepannulus en de abdominale aorta veroorzaakt non-immunologische degeneratie in het weefsel. End-to-side anastomose brengt uitkomst voor dit diameter probleem.

100% overleving en doorgankelijkheid hebben wij geconstateerd in de aortaklepimplantaten op dag 21 na implantatie. De klepfunctie is bestudeerd door retrograde injectie. Allogene kleptransplanten waren allen incompetent op dag 21. Syngene transplantaten vertoonden geen fibrose of intima-verdikking en waren allen competent. Dit transplantatiemodel vermindert niet alleen de niet-immunologische factoren van klepdegeneratie maar maakt het ook mogelijk de klepfunctie aanvullend te analyseren. Het bestuderen van het effect van immuunactivatie op klepdegeneratie en dysfunctie wordt hierdoor mogelijk.

Hoofdstuk 8: Hartklep dysfunctie door cellulaire afstoting: een studie met een nieuw heterotoop transplantatiemodel in de rat. De relatie tussen immuun-gerelateerde weefseldegeneratie en klepdysfunctie is bestudeerd in een heterotoop aortaklep transplantatiemodel. In een serie van 10 allogene (WAG-BN of WAG-DA) en 10 syngene (WAG-WAG of DA-DA) verse aortaklep transplantaties werden de klepfunctie en (immuun)histologische veranderingen onderzocht 21 dagen na transplantatie. 80% van de allogene kleppen en 10% van de syngene kleppen waren incompetent. Allogene klepbladen waren gedeformeerd en a-cellulair. Infiltraten met $CD4^+$, $CD8^+$ T-lymfocyten en $CD68^+$ macrofagen werden gezien in de vaatwand. Syngene kleppen hadden normale klepbladen zonder fibrose of intima-verdikking. Een minimum aan T-lymfocyten en macrofagen werden aangetroffen in adventitia en de myocardrand. Deze studie bewijst dat transplantatie van verse aortakleppen tussen verschillende rattenstammen resulteert in afstoting van het transplantaat, wat leidt tot klepincompetentie.

Hoofdstuk 9: Donor-reactieve helper T-lymfocyten frequenties correleren met afstoting van verse aortakleppen in de rat. In hoofdstuk 8 is beschreven dat verse allogene aortakleppen worden afgestoten na implantatie. Na klinische implantatie van donorhartkleppen wordt in het bloed van bijna alle patiënten een toename gezien in de frequentie HTLp (hoofdstuk 4). Een direct verband tussen toename van de HTLp frequentie en afstoting van de klep is nog niet gelegd omdat in de praktijk na implantatie geen routinematig histologisch onderzoek kan worden uitgevoerd op de donorklep. In deze studie is de waarde van de HTLp frequentie analyse als indicatie voor klepafstoting onderzocht. In het bloed en in de milt zijn de HTLp frequenties bepaald op dag 7 en dag 21 na de transplantatie van verse allogene (WAG-DA) en syngene (DA-DA) aortakleppen. Huidtransplantaties werden verricht als positief controleexperiment. Op dag 7 en 21 werden ook de kleppen onderzocht op competentie en histologie. De donor-reactieve HTLp frequentie nam toe in de milt, 7 dagen na allogene transplantatie. Toename van HTLp werd in het bloed pas na 21 dagen waargenomen. De competentie van allogene aortakleppen daalde van 71% op dag 7 naar 0% op dag 21 ($p=0.01$). Alle syngene kleppen bleven daarentegen competent. Op dag 7 waren alle allogene klepbladen en vaatwanden geïnfiltreerd met mononuclaire cellen en op dag 21 waren de klepbladen gedeformeerd. In de syngene aortakleppen vonden we weinig mononucleaire infiltraties, mogelijk als gevolg van de

chirurgische ingreep. Transplantatie van verse allogene aortakleppen induceert een toename in donor-reactieve HTLp frequenties. Dit correleert met vroegtijdige histologische tekenen van afstoting en latere klepdysfunctie. Het monitoren van donor-reactieve HTLp in de ontvangers van donorkleppen is een goed alternatief voor het signaleren van afstoting van het transplantaat, dat uiteindelijk kan resulteren in klepdysfunctie.

Hoofdstuk 10: Immunologische aspecten van verse en gecryopreserveerde aortakleptransplantatie in de rat. Omdat immunogeniciteit van allogene transplantaten is gerelateerd aan de expressie van allogene antigenen, kan verondersteld worden dat cryopreservatie invloed heeft op de immunogeneiciteit van donorhartkleppen. In deze studie hebben we het effect van cryopreservatie op de klepfunctie, histomorfologie en weefsel-immunogeniciteit onderzocht. Verse (FA) of gecryopreserveerde (CA) allogene en verse (FS) en gecryopreserveerde syngene (CS) aortakleptransplantaties werden verricht. De competentie en immuun-histologie van de kleppen werden onderzocht op de 7e en 21e dag na transplantatie. Allogene reactiviteit werd geanalyseerd door middel van concanavaline A stimulatie en frequentie analyse van donor-reactieve HTLp in het bloed en in de milt. Verse en gecryopreserveerde allogene hartkleppen vertoonden een progressieve incompetentie en mononucleaire infiltraties terwijl verse syngene kleppen competent bleven en geen afstotingsverschijnselen lieten zien. Cryopreservatie veroorzaakt een vroegtijdige incompetentie van syngene en allogene kleppen door de vorming van retrovalvulaire thrombi. De reactiviteit van T-lymfocyten en toename van HTLp frequenties in het bloed en in de milt waren significant hoger na allogene transplantaties (FA+CA) in vergelijking met syngene transplantaties. Een lagere allogene reactiviteit werd geconstateerd na transplantaties van gecryopreserveerde kleppen. Deze studie bevestigt de immunogeneiciteit van zowel verse als gecryopreserveerde allogene aortakleppen. Inductie van allogene reactiviteit is gerelateerd aan histologische tekenen van afstoting. Het verlies van structurele integriteit van allogene kleppen leidt vervolgens tot klepfalen. Cryopreservatie vermindert, maar elimineert niet de immunogeniciteit van allogene kleppen. Tevens maakt dit de kleppen meer thrombogeen door chemische of mechanische factoren. Bij extrapolatie van deze resultaten naar de klinische situatie, kan het volgende worden gesteld: implantatie van een gecryopreserveerde allogene hartklep induceert een donor-specifieke cellulaire immuun-activatie. Deze immuun-activatie resulteert in vroegtijdige structurele weefselbeschadiging, wat vervolgens op een later tijdstip kan leiden tot klepfalen.

Hoofdstuk 11: Discussie In dit hoofdstuk worden de belangrijkste resultaten en conclusies uit de verschillende studies nader besproken in het licht van de eerder gestelde doelen. Mogelijke klinische implicaties gebaseerd op diverse conclusies en aandachtspunten voor toekomstig onderzoek worden uiteengezet. Het blijft moeilijk een definitieve uitspraak te doen over het belang van matching voor transplantatie antigenen in het kader van donorhartklepimplantaties. Klinische matching voor

transplantatie antigenen is vrijwel onmogelijk vanwege de diversiteit van het HLA systeem en de tekorten aan donorhartkleppen. Modulatie van de immunogeniciteit van de donorkleppen lijkt beter haalbaar. Cryopreservatie is op zichzelf al een vorm van modulatie. Veel aandacht gaat uit naar "tissue engineering" waarbij wordt gestreefd naar een biologische klepprothese met een maximale levensduur en een minimale immunogeneiciteit. Door *in vitro* pre-incubatie met lichaams-eigen-cellen van de ontvanger ontstaat een viabele, autologe bioprothese. Dit veelbelovende concept verkeert momenteel nog in de preklinische fase. Het identificeren van patiënten met een hoog risico voor vroegtijdig klepfalen is belangrijk omdat additionele immunosuppressieve therapie met moderne farmaca mogelijk de versnelde degeneratie kan afremmen. Prospectief gerandomiseerde experimenten met nieuwe immunosuppressiva die minder bijwerkingen kennen en waarin deze verstrekt worden binnen een beperkt tijdsinterval kunnen mogelijk leiden tot een verbeterde duurzaamheid van donorhartkleppen zonder de nadelige gevolgen van de klassieke langdurige hoge dosis immunosuppressieve therapieën.

Dankwoord

Een proefschrift is niet compleet zonder een afsluitend dankwoord. Dit deel van het proefschrift is mateloos populair onder de lezers omdat het aanvullende informatie geeft over de manier waarop, met welk gevoel en vooral met wie de auteur het onderzoek heeft voltooid. Want de weg naar een promotie is vaak eenzaam maar nooit alleen af te leggen. Daarom rest mij nog een ieder te bedanken die actief dan wel passief heeft bijgedragen aan de totstandkoming van dit proefschrift. Zonder iemand te vergeten wil ik graag enkele personen bij naam noemen.

Als eerste wil ik mijn beide promotors bedanken. Hooggeleerde professoren A.J.J.C. Bogers en W. Weimar. Beste Willlem en Ad, vier jaar na dato weet ik eindelijk ook wat promoveren inhoudt maar nu is het proefschrift klaar. Inderdaad kent het wetenschappelijk onderzoek pieken en dalen. Maar wanneer lastige problemen zich voordeden, waren jullie altijd bereid (ook zonder afspraak) deze te bespreken en wisten jullie mij telkens weer te motiveren. Ook dank ik jullie voor het vertrouwen dat jullie in mij hebben gesteld en de vrijheid die jullie mij hebben gegeven ten tijde van de uitvoering van het onderzoek.

Mijn directe begeleider dr. L.M.B. Vaessen en mijn naaste collega gedurende dit project, dr. M.J.P. Welters-Schoenmaekers. Beste Len, jij heb de dagelijkse supervisie van het hartkleppenonderzoek na dr. Jutte op je genomen. Ik heb vanaf de eerste dag van jouw enthousiasme voor wetenschappelijk onderzoek in het algemeen en jouw ervaring en expertise op het gebied van transplantatie immunologie in het bijzonder veel geleerd. Ik dank je voor je introductie en begeleiding door de vaak complexe materie van de immunologie. Beste Marij, we hebben gedurende dit project vele ups en enkele downs gekend in de uitvoering van de experimenten. Die uurtjes achter de flow-kast zijn zeker niet voor niets geweest. Gedurende dit hartkleppen-project hebben wij met z'n drieën vele vruchtbare discussies gevoerd maar helaas heeft dit niet kunnen leiden tot een voortzetting van het project in Rotterdam. Ik hoop dat je tijdens je volgende project minimaal net zo veel succes heb als hier. Dr. R.L. Marquet, beste Richard, jou wil ik met name bedanken voor je inspirerende belangstelling voor het onderzoek en de gastvrijheid waarmee ik werd ontvangen op het laboratorium voor experimentele chirurgie. Tevens dank ik je voor het aanvaarden van de functie van secretaris van de kleine commissie.

Aanvullend wil ik graag Prof. dr. F.H.J Claas en Prof. dr. B. Mochtar bedanken voor hun betrokkenheid bij dit project en hun aanvaarding om zitting te nemen in de kleine commissie.

De overige leden van de promotie commissie, Prof. dr. Helbing, Prof. dr. M.L. Rose en Dr. J. Prop wil ik bedanken voor hun bereidheid tot deelname. Dear professor Rose, I would like to thank you for your participation in the committee and I'm pleased with your presence during the ceremony.

Dr. C.C. Baan, beste Carla, jou wil ik bedanken voor je betrokkenheid bij dit project. Je adviezen zijn altijd welkom geweest en ik heb met veel plezier naast je aan het bureau gezeten. Dr. F.M. Hoekstra, beste Franciska, zonder jouw "voorzet" was dit proefschrift er zeker niet gekomen. Barbara, Kees,

Ronella, Hester, Cecile, Paula, Chris, Lisette, Wendy, Annemiek, Maud, Saskia, Thea, Wenda en ook stagiaires op het lab Interne geneeskunde I wil ik bedanken voor de gezelligheid en vooral voor die lekkere negerzoenen! Willy Zuidema en Saskia Klomp wil ik bedanken voor hun behulpzaamheid.
De internisten, Teun van Gelder, Iza van Riemsdijk, Bob Zietze, Peter Smak Gregoor, Joke Rootnat en Jacqueline Rischen wil ik graag bedanken voor hun blijvende belangstelling voor de voortgang van mijn experimenten.
Zonder hulp vanuit de hartkleppenbank Rotterdam was de inhoud van dit proefschrift niet volledig geweest. Dr. A.P.A. Stegmann, beste Sander, met jou heb ik aardige discussies gevoerd over de donorhartkleppen. Met name de ontwikkelingen rondom de preservatiemethoden van donorkleppen was vaak het onderwerp van gesprek. Jammer dat onze samenwerking in Rotterdam niet gecontinueerd kan worden, maar hoop dat je in je nieuwe functie net zoveel succes en plezier zult hebben. Overige leden van de hartkleppen bank, Zohara, Corina en Alice wil ik bedanken voor de continue toestroom van onderzoeksmateriaal.
Behalve op het lab Interne I heb ik vele uren doorgebracht op het lab van de Experimentele chirurgie. Fred, van jou heb ik microchirurgische technieken mogen leren en ik heb zeker in de toekomst daar veel profijt van. Het "Rotterdam-model" was zonder jouw hulp er nooit gekomen.
Dr. Ron de Bruin, R de B, bedankt voor je interesse in dit project. Bij jou kon ik altijd langs voor bruikbare adviezen of gewoon gezellig "ouwehoeren".
Ook wilde ik patholoog Pieter Zondervan bedanken voor het beoordelen van de histologische coupes en het mogelijk maken om histologisch onderzoek te doen op de afdeling pathologie.
De thoraxchirurgen, Lex van Herwerden, Lex Maat, Jos Bekkers, Peter de Jong, Charles Kik, John BolRaap, Arie-Pieter Kappetein en Robert Klautz en de andere collega arts-assistenten van de afdeling Cardio-Thoracale chirurgie, ik dank jullie allen voor jullie belangstelling voor mijn promotieactiviteiten en jullie collegiale opstelling om de laatste loodjes enigszins te verlichten.
Daarnaast dank ik de kindercardiologen voor het verzamelen van patienten materiaal. Verschillende experimenten zouden niet uitgevoerd kunnen worden zonder de medewerking van de patiënten. Het is onvoorstelbaar wat men uit een buisje bloed kan halen.
Nicole van Besouw en Jeroen Lijmer, bedankt dat jullie mij bij willen staan op 14 november, jullie weten beiden in ieder geval wat mij die dag nog te wachten staat. Laten we de dag vervolgens afsluiten met een groot feest.
Lieve vrienden en familie, ik dank jullie allen voor jullie belangstelling en steun ten tijde van mijn onderzoeksperiode. Ik stel jullie eindeloos geduld en tolerantie erg op prijs.
Papa en Mama, bedankt voor jullie inzet om mij de mogelijk te bieden te studeren. Nooit hebben jullie aan mijn capaciteiten getwijfeld en hebben jullie altijd voor mij klaar gestaan.
Mijn liefste Älskling, Carla, zonder jou had ik dit proefschrift misschien wel kunnen voltooien maar met jou samen is het zo oneindig veel leuker. Jouw onvoorwaardelijke steun en vertrouwen heb ik heel erg gewaardeerd. Ik heb je lief!

Curriculum Vitae

Frans Bing Sien Oei is geboren op 28 september 1970 in Fukien te China. Zijn VWO-diploma behaalt hij in 1988 aan het Hervormd Lyceum Zuid in Amsterdam. In november 1995 legt hij succesvol zijn artsenexamen aan de Universiteit van Amsterdam af. In de doctoraalfase van zijn studie geneeskunde gaat zijn belangstelling uit naar de cardiologie. Dit resulteert in deelname aan "The International Medical Summer School" (juli 1991) onder begeleiding van Prof. dr. M.J. Janse, verbonden aan het Academisch Medisch Centrum, waarin recente ontwikkelingen in cardiovasculaire ziekten centraal staan. Ook in zijn afstudeerscriptie besteedde hij aandacht aan een onderwerp op het terrein van de cardiologie: Thermal damage of vascular tissue by the Excimer laser. Het onderzoek voert hij uit onder begeleiding van dr. J. Hamburger en Prof. dr. M. van Gemert in het lasercentrum van het Academisch Medisch Centrum. Tijdens zijn co-schappen verlegt hij zijn interesse naar de snijdende specialismen.

Deze combinatie van verschillende interesses leidt tot de keuze om na het artsexamen, in maart 1996, als agnio cardio-thoracale chirurgie aan de slag te gaan in het Academisch Ziekenhuis Maastricht, na een korte periode werkzaam te zijn geweest als senior house officer in het Victoria General Hospital in Blackpool te Groot Brittanie. In het Academisch Ziekenhuis Maastricht werkt hij onder dr. K. Prenger, plaatsvervangend opleider.

In juni 1997 start hij zijn promotieonderzoek in Rotterdam. Het onderzoek is een gezamenlijk project van de afdelingen cardio-thoracale chirurgie (Prof. dr. A.J.J.C. Bogers) en inwendige geneeskunde I (Prof. dr. W. Weimar) in het Academisch Ziekenhuis te Rotterdam. Sinds april 2001 is hij werkzaam als arts-assistent, niet in opleiding, op de afdeling cardio-thoracale chirurgie in ditzelfde academische ziekenhuis.

Medio 2002 zal hij hier beginnen aan zijn opleiding tot cardio-thoracaal chirurg.

List of publications

Oei FBS, Welters MJP, Bonthuis F, Vaessen LMB, Marquet RL, Zondervan PE, Weimar W, Bogers AJJC. A size-matching heterotopic aortic valve implantation model in the rat. **J Surg Res. 1999;87:239-244**

Oei FBS, Welters MJP, Vaessen LMB, Marquet RL, Zondervan PE, Weimar W, Bogers AJJC. Heart valve dysfunction due to cellular rejection in a novel heterotopic transplantation rat model.**Transplant Int.** 2000;13[S13]:S528-531

Oei FBS, Welters MJP, Knoop CJ,Vaessen LMB, Stegmann APA, Bogers AJJC, Weimar W. Circulating donor-specific cytotoxic T lymphocytes with high avidity for donor human leukocyte antigens in pediatric and adult cardiac allograft valved conduit recipients. **Eur J Card Thorac Surg** 2000; 18:466-72

Oei FBS, Welters MJP, Vaessen LMB, Stegmann APA, Bogers AJJC, Weimar W. Induction of cytotoxic T lymphocytes with destructive potential after cardiac valve allograft implantation. **J Heart valve Dis.** 2000; 9:761-68

Gerestein CG, Takkenberg JJM, Oei FBS, Cromme-Dijkhuis AH, Spitaels SEC, van Herwerden LA, Steyerberg EW, Bogers AJJC. Right ventricular outflow tract reconstruction with an allograft conduit. **Ann Thorac Surg** 2001;71:911-8

Oei FBS, F.J.M.F. Dor, Stegmann APA, Welters MJP, Vaessen LMB, Marquet RL, Bogers AJJC, Weimar W Helper T cell frequencies after implantation of aortic valve allografts in rats **Transplantation Proceedings** 2001; 33:640-41

Welters MJP, Oei FBS, Vaessen LMB, Stegmann APA, Bogers AJJC, Weimar W. Increased numbers of circulating donor-specific T helper lymphocytes after human heart valve transplantation. **Clin Exp Immunol** 2001; 123:1-7

Oei FBS, Stegmann APA Vaessen LMB, Marquet RL, Weimar W, Bogers AJJC. Immunological aspects of fresh and cryopreserved aortic valve transplantation in rats. **Ann Thorac Surg** 2001;71:S379-84

Dor FJMF, Oei FBS, Vaessen LMB, Marquet RL, Bogers AJJC , Weimar W. Frequencies of donor reactive helper T lymphocytes correlate with rejection of fresh allogeneic aortic valve grafts in rats. **J Heart valve Dis.** *In press*

Oei FBS, Stegmann APA,. van der Ham F, Zondervan PE, Vaessen LMB, Baan CC, Weimar W and Bogers AJJC. The presence of immune stimulatory cells in fresh and cryopreserved donor aortic and pulmonary valve allografts **Submitted to Journal of Heart Valve Disease**