THE IMMUNOGENICITY OF HUMAN CARDIAC VALVE ALLOGRAFTS IN VITRO AND IN VIVO

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DE IMMUNOGENICITEIT VAN HUMANE HARTKLEPTRANSPLANTATEN IN VITRO EN IN VIVO

PROEFSCHRIFT

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CONTENTS

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Chapter 1

IMMUNOLOGICAL PHENOMENA AFTER CARDIAC VALVE TRANSPLANTATION: REVIEW OF THE LITERATURE

This chapter is based on:

"The activation of the immune system by human cardiac valve allografts", accepted for publication in:
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1.1 Introduction.

Allogeneic transplantation has become an accepted method for the replacement of diseased organs and tissues. The concept of cardiac valve transplantation for the replacement of semilunar valves was introduced by Lam et al in 1952[1]. In about 1960 the first human cardiac valve allografts were implanted[2,3]. Human donor valves have become a good alternative for other valve substitutes (porcine valve prostheses and mechanical valves) because of their superior hemodynamic performance and the absence of post-operative thrombosis and thromboembolism. Further, these valves are relatively resistant to endocarditis and recipients of human donor valves do not require anticoagulant therapy.

At this moment, the valve allografts are stored at heart valve banks. In 1995, 68 heart valve banks were known world-wide[4]. Before the introduction of cryopreservation techniques, human valve allografts were stored at 4°C in a nutrient medium containing an antibiotic solution, with a maximum storage time of 6 weeks[5,6]. These antibiotic-sterilized, "fresh wet-stored" allografts showed a better medium-term (7-10 years) clinical performance than glutaraldehyde-treated human valves[7,8]. Alternative storage techniques (freeze-drying, irradiation) resulted in a shorter long-term graft survival compared to mechanical valves[9-11] and a higher incidence of cusp rupture[12]. The introduction of cryopreservation procedures offered long-term storage and improved the availability of the valves.

In The Netherlands, cryopreserved human valves are stored at the Heart Valve Bank, Thorax Center, University Hospital Rotterdam-Dijkzigt. The aortic and pulmonary valves are obtained from heart beating donors or from non-heart beating donors within 24 hours after death. At this moment, contraindications for valve donation are donor age older than 55 years, sepsis, congenital heart anomalies and heart valve diseases, myocarditis, hypertrophic obstructive cardiomyopathy or previous cardiac surgery, Marfan's disease and malignancies. Patients with a positive screeningstest

for Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus (HIV) or lues are also excluded[13]. World-wide there is no agreement on the minimum acceptable age of valve donation[4]. The hearts are explanted under aseptical conditions by a surgical team of Bio Implant Services (BIS). Thereafter, the valves are prepared at the Heart Valve Bank under sterile conditions. Before the cryopreservation procedure, the allografts are incubated into a low concentration antibiotic solution for 24 hours at 4°C. Dimethylsulfoxide (DMSO) is used to reduce the damage caused by freezing. Thawing occurs according to the standard thawing protocol of the Heart Valve Bank at the day of valve implantation.

1.2 Clinical valve allograft survival.

In general, the precision of valve allograft insertion has been regarded as the most accurate predicting factor for early and long-term graft survival[14]. Factors which are significantly associated with valve allograft failure are younger age of the recipient at operation, low weight of the recipient, young donor age, small graft size and a cryopreservation time less than 24 hours[15,16]. However, the variability in patient age, surgical techniques, perioperative mortality and preservation techniques interfere with the possibility to make a valid comparison between different follow-up studies. Long-term follow-up studies after implantation of cryopreserved valves show good clinical results, especially in adult recipients[17,18]. Nevertheless, valve deterioration resulting from an intrinsic abnormality of the graft (e.g. leaflet tear, calcification, stenosis or stent creep) is frequently reported. This **structural valve failure**, which is not related to technical factors as sizing and surgical procedures, infection or thrombosis as determined by reoperation, is frequently seen[19]. Structural valve deterioration does not necessarily lead to valve dysfunction[20-22]. However, some studies give rise to concern about the incidence of graft failure, especially early valve allograft

which is frequently observed in young recipients[23-25]. Freedom from reoperation of cryopreserved valves placed into the right ventricular outflow tract in children only ranges from 55-80% at 4 to 6 years after implantation[15,16,26-28]. Baskett et al examined the function of cryopreserved pulmonary valve allografts echocardiographically in 44 children (mean age at implantation: 6.2 years, range: 3 days to 20.2 years). After 50 months, freedom from echocardiographic graft failure was 44%. In this study, graft failure was defined as more than 50 mmHg pulmonary regurgitation[15]. In our center, 189 patients (mean age 16 years, range: 2 weeks - 54 years) with an allograft in the right ventricular outflow tract were followed for 2.5 years (mean, range: 4 weeks to 9 years). In this study, the patient survival at 5 years was 91% and freedom from structural valve failure was 83%[16]. Many authors suggested that structural valve failure could have an immunological basis, because valve donor and acceptor are not matched for ABO blood group antigens or the transplantation antigens, the Major Histocompatibility Complex (MHC) antigens, while immunosuppressive therapy is not routinely administered to valve recipients. The antigenicity of cardiac valve allografts has been a matter of debate since their introduction for clinical use. Fresh and cryopreserved valves have classically been regarded as tissues with low antigenicity because of the relatively good medium-time clinical performance without immunosuppression.

In this chapter, the immunological aspects of cardiac valve transplantation in animal studies (sensitization of the recipients, histological and immunohistochemical findings of explanted valve allografts in vivo) are discussed. Further, results of studies directed at the immunogenicity of human cardiac valve allografts, the effect of the ABO blood group compatibility between valve donor and recipient on valve allograft survival and some aspects of immunomodulation and immunosuppression are expounded.

1.3 Immunogenicity of allogeneic valves in animal studies.

To analyse the antigenicity of fresh and cryopreserved allogeneic cardiac valves in vivo, various animal models have been used. These studies mainly aimed at sensitization of recipients, histological signs of rejection after implantation of allogeneic valves and on the possible correlation between immunological activity against the graft and valve insufficiency.

Sensitization of valve recipients was examined by Mohri et al. They showed that allogeneic skin grafts were rapidly rejected when dogs were sensitized by subcutaneous implantation of a valve of the same donor[29]. In contrast, when a valve instead of a skingraft was transplanted in the orthotopic position in these sensitized animals, no acute rejection was observed. Histological examination showed only few polymorphonuclear leukocytes and mononuclear cells in an intact valve stroma. No significant differences were found in the functional durability of orthotopically transplanted leaflets in either sensitized or nonsensitized dogs. However, plasma cells and lymphocytes infiltrating the allogeneic implants were observed more than 2 months after transplantation. The authors suggested that this late cellular rejection could be the result of the low antigenicity of the aortic valve. This conclusion is not in line with their findings that these valves are able to sensitize their experimental animals as they showed by the rapid rejection of skin grafts. An alternative explanation could be that these transplanted valves are not instantly recognized, not even by a sensitized immune system, suggesting that the implantation procedure temporarily affected the antigenicity of the valve. The relative lack in immune response is, however, not an uniform finding.

Baue and collegues examined the immunologic response to heterotopic fresh aortic valve transplants in nonsensitized calves and animals presensitized with donor skin[30]. Destruction of the allogeneic valves after 7 days of transplantation was found in all instances. No histological differences in the explanted valve allografts were found between the

presensitized and nonsensitized animals. In this study, the histological findings were comparable to those of rejecting organ transplants, although the acute rejection process did not result in dysfunction of the transplanted valves.

Buch et al examined histological differences between transplanted allogeneic and autologous valve allografts in dogs after various months after transplantation[31]. In the allogeneic explanted valves, structural alterations were observed already 5 days after implantation. Absence of endothelium and a zone of acellularity just below the surface of the valves was observed, while autologous grafts retained the normal endothelial lining. Three months, 6 months and 1 year after implantation, both homologous and autologous valves were moderately thickened due to hypercellularity. The explanted grafts showed fibroblast hyperplasia and areas of acellularity, but infiltrates were not detected. Despite the histological evidence of valve damage, these valves retained their function during this experimental study[31].

In a study on rats, Thiede et al examined the level of sensitization after transplantation of one versus two allogeneic valve leaflets. The valves were implanted intravascularly and the time to skin rejection was measured. One heart valve leaflet caused strong sensitization, resulting in accelerated skin rejection. There was no statistically significant difference in the moment of rejection between rats that received one or two valve leaflets, showing that the quantity of valve antigen was not able to either enhance or suppress the antigeneic process. In this report, the degree of sensitization varied significantly according to the degree of histoincompatibility[32], which was also observed by Khatib et al in a comparable study[33]. Lupinetti et al examined the effect of immunological different rat strains on aortic valve allograft calcification[34]. These authors were not able to demonstrate a correlation between the degree of histoincompatibility and calcification on scanning electron microscopical findings, although syngeneic valves showed less calcified areas than allogeneic valves in this model[34]. Immunogenetic factors

were also of importance in a study by Gonzalez-Lavin et al, who showed that transplantation of valves between genetically related dog combinations was associated with significantly less degenerative changes compared to histological findings in valves transplanted between genetically different dogs[35].

Zhao et al examined the immune response to MHC antigens after implantation of an allogeneic valve in different rat strains[36]. The proliferative response of acceptor lymphocytes against donor spleen cells was measured before and after implantation of the allogeneic valve using mixed lymphocyte cultures. The authors demonstrated a donor-specific stimulation one month after implantation. The same group also observed an increase of donor-specific cytotoxic T-lymphocyte frequencies in a limiting dilution assay with splenocytes, accompanied by an increase in the level of anti-donor antibodies[36].

In conclusion, these animal studies show that implantation of allogeneic valves is followed by a specific donor-directed immunological response. Histological signs of acute and chronic rejection, especially in the allogeneic grafts are generally observed, although these findings do not always correlate with valve dysfunction. Autologous valves show less signs of inflammation than allogeneic valves, and the degree of the immune response is related to the degree of histoincompatibility in some animal models presented. These results suggest that matching for MHC antigens can result in a decreased immune response against the donor valve.

1.4 Immunogenicity of human valves.

Target antigens: ABO blood group and Major Histocompatibility Complex antigens.

ABO blood group antigens could play a role in valve allograft rejection. The effect of compatibility for ABO blood group antigens between valve donor and acceptor on graft survival has sparsely been reported in the literature, mainly in retrospective studies. Some investigators have carried out ABO matching and even recommend ABO matching[22,37-39]. In other studies, a relation of ABO blood group incompatibility and valve allograft failure could not be found[15,38,40]. The relation between valve insufficiency and blood group incompatibility approached significance (p=0.05) in the echocardiographic follow-up study of young children by Baskett et al[15]. In contrast, Balch and collegues could not demonstrate a correlation between ABO compatibility and long-term valve allograft survival[41]. In this study, the majority of the patients **with** valve failure (130/188 patients) were ABO compatible. Regardless of this finding, the authors mentioned the presence of denuded endothelium, fibrin deposits, calcification and mononuclear infiltrates in unsuccessful aortic valve transplants, suggesting a role for other antigens than the ABO blood group system in evoking an immune response. The cell surface antigens coded by the Major Histocompatibility Complex (MHC) are obvious candidates. The presence of these so-called transplantation antigens is a prerequisite for the initiation of an immune response against foreign tissue. In humans, the Major Histocompatibility Complex molecules are called the Human Leucocyte Antigens (HLA). The HLA class I molecules (HLA -A, -B and -C) are membrane glycoproteins, which can be expressed by many nucleated cells. HLA class II molecules (HLA -DR, -DQ, and -DP) are present on antigen presenting cells (dendritic cells, Langerhans' cells), B-lymphocytes and activated T-lymphocytes[42]. In valve transplantation, the viability of fibroblasts and endothelial cells is considered to be an important

factor contributing to immunostimulation, because both fibroblasts and endothelial cells cultured from valve allografts have shown to be able to express Human Leucocyte Antigens (HLA) class I and class II antigens. Salomon et al examined the expression of HLA class II antigens on fibroblasts cultured from cryopreserved aortic valves after incubation with Interferon-gamma[43]. The authors observed an upregulation of class II antigens on the majority of fibroblasts. Yacoub et al also examined the presence of HLA-antigens in different components of human cardiac valves. The valve allografts were studied by staining with monoclonal antibodies and the influence of sterilization procedures was investigated[44]. Before storage, valve allografts showed weak staining for class I antigens on endothelial cells, which gradually disappeared within 48 hours during storage in Hartmann's solution. Within the matrix, class I positive leucocytes were observed which also disappeared within 48 hours. Class II staining of endothelium was negative both before and after storage, but positive for cells just below the endothelium, possibly representing the presence of leucocytes or dendritic cells. As endothelial cells form the first barrier between the allo-reactive immune system and the donor valve, these cells could play an important role in the initiation of the immune response against the donor valve. To analyze the immunogenicity of valve endothelium in vitro, Simon et al cultured endothelial cells from fresh valve leaflets[45], according to the isolation method of Johnson and Fass[46]. Mixed cell cultures with valve endothelium as stimulator and lymphocytes as responder cells resulted in high proliferative responses. A very potent antigen presenting cell which could be present in a valve before implantation is the dendritic cell. This cell expresses various surface markers including HLA class II antigens, complement receptors and adhesion molecules. These cells are difficult to identify, because many other cells like fibroblasts, lymphocytes and macrophages stain positive after incubation with these markers. The nerve tissue protein S-100 stains a wide group of dendritic cells, but does not stain

fibroblasts, lymphocytes and macrophages[47,48]. Bobryshev and Lord used this marker to identify dendritic cells into the arterial intima, because the intima is not innervated and is not supposed to contain other S-100 positive cells. They indeed detected areas of the arterial wall with S-100 positive cells[49]. Because human valve allografts also contain an aortic or pulmonary root, dendritic cells could play a role in the antigen presentation after valve allograft transplantation. Schoen et al examined cryopreserved valve allografts which had to be explanted because of growth-related conduit or valve stenosis. They concluded that the function of cryopreserved valves is not related to the presence of viable cells, but to preserved collagen[50]. Immunohistochemical studies of these explants showed that inflammatory cells, primarily T-lymphocytes, were present in only one valve (out of 20 valves examined), while viable fibroblasts or endothelial cells could not be detected.

Immunological response.

By staining with monoclonal antibodies, Yankah et al identified activated complement (C3C) and immunoglobulins on the surface of explanted allogeneic valves 4 weeks after implantation[38,51]. In these studies, the immunological findings were not related to valve failure. In a comparable study by Lupinetti et al more or less the same results were obtained[52]. To follow the immunological process after allogeneic valve implantation, cytoimmunological monitoring has been used by Schutz et al[53]. This group examined 16 patients who had received ABO compatible (n=9) or ABO incompatible (n=7) cryopreserved aortic valves. An immunological reaction could be detected in all patients 5 days after implantation. The increased activation index spontaneously disappeared after 7 days without the use of immunosuppressive drugs, but in the ABO compatible group a prolonged activation was observed. Echocardiography as postoperative function control 3 months after implantation showed hemodynamically irrelevant valve

insufficiency in both groups. The authors conclude that transplantation of cryopreserved allogeneic valves leads to an immunological reaction in the early postoperative course due to T-cell activation which is reversible, and that ABO incompatibility does not affect the echocardiographic outcome after 3 months. Although in our opinion cytoimmunological monitoring is a method which is too nonspecific to diagnose rejection after organ transplantation[54], it may certainly reflect nonspecific immunological reactivity in peripheral blood after valve transplantation.

The detection of allo-antibodies after implantation of valve allografts could give information about the presence of immunogeneic Human Leucocyte Antigens in the graft. Davies et al examined serum of patients who received a freeze-dried allogeneic valve, but they failed to detect antibodies[55].

1.5 Modulation of valve antigenicity.

Studies in animals and humans after cardiac valve implantation which are focussed on signs of rejection show different results. An explanation for these different results could be the varying preservation methods, which can lead to a change in the cellular composition of graft, resulting in modification of the antigeneic load. A reduction of the antigenicity of the transplant could lead to a less aggressive immune response of the acceptor. Reducing the number of cells expressing transplantation antigens, as mentioned on the preceding pages, is an option, provided that the function of the valve leaflets remains unaffected. Preservation methods can therefore be considered as forms of immunomodulation. It has been reported by El Khatib et al that fresh aortic valve allografts contain more viable endothelial cells than allografts stored at 4°C in an antibiotic solution, leading to the loss of antigeneic load[56]. Transplantation of allogeneic valves between rat strains resulted in a shorter time to skin graft rejection when fresh valves were transplanted, compared to rats that received an allograft preserved for 21

days in a nutrient medium[56]. Lang et al detected viable endothelial cells on cryopreserved valves (and no viable fibroblasts)[57], while van de Kamp et al reported the presence of viable fibroblasts and an intact structure of collagen fibrills after sterilization and controlled freezing of aortic valves[58]. Brockbank et al confirmed an enhanced fibroblast viability after cryopreservation and storage at -135°C up to 2 years by measuring the glycine uptake[59], but valves stored at -80°C showed loss of fibroblast viability after 1-6 weeks[59]. The group of Lupinetti used immunofluorescence staining technics to show that endothelial cells were present on the surface of the valve leaflets from 16% of 131 cryopreserved aortic valve allografts[60]. Yankah et al also demonstrated the presence of viable endothelial cells on the valve surface after cryopreservation, while on fresh sterilized valves these cells were absent[61]. These studies show that the presence of endothelium on cryopreserved valves may vary. To our opinion, this can be explained by the use of different freezing technics and antibiotic sterilization procedures. Nevertheless, endothelial cells could be important initiators of the allogeneic immune response, and in studies on the immunogenicity of vascular endothelium, these cells even have shown to be a major target of cell mediated immune-injury[62-65]. Moreover, both cytotoxic T-cells and allo-antibodies specifically directed at endothelial cell surface molecules have also been detected in explanted rejected organs[66-68].

Long-term follow-up studies of patients in the clinic show a marked improvement with the transplantation of cryopreserved valve allografts compared to fresh valves[17], suggesting that the cellular composition, the collagen structure or antigenicity of valve allografts is altered by cryopreservation. Cochran used Fisher rats to study the effect of cryopreservation on the antigen expression of aortic allografts[69]. Allogeneic aortic valves were implanted subcutaneously and the time of skin graft rejection (first and second set reaction) was compared between recipients of fresh and cryopreserved valves. No statistical difference was found between

fresh and cryopreserved valves for first and second set rejection phenomena, indicating no effect of cryopreservation.

Calhoun examined the difference between survival of fresh and cryopreserved venous allografts in genetically characterized dogs[70]. In contrast to Fisher, he detected early deterioration of the intima of cryopreserved venous grafts while the fresh venous transplants were histologically normal. It remained unclear why cryopreservation was associated with early thrombosis and transplant failure in this study. Nevertheless, cryopreservation and/or thawing could damage the intima of the venous allografts, leading to thrombotic complications.

Jonas et al compared hemodynamic, angiografic and histological findings in a long-term sheep model after implantation of a cryopreserved versus an antibiotic sterilized aortic valve allograft[71]. The authors found no significant difference in transconduit gradient or histological findings 9 months after transplantation, but calcification was prominent within the conduit wall of all animals.

Modulation of alloreactivity by phenotypic manipulation of donor endothelium has been performed on heart allografts in rats[72]. Therefore, host endothelium was isolated and perfused into donor allografts. Allografts pretreated with host-endothelium survived for 12 days, while untreated heart allografts survived 7 days. This method could become an alternative method to reduce the immunogenicity of valve allografts, because endothelial cells can be easily isolated[73] and valve allografts could remain ex vivo for more extended periods than heart transplants to allow attachment of the donor endothelium on the graft. Another alternative could be the pretreatment of allografts with cyclosporine, which has been earlier performed on venous allografts in dogs. This treatment resulted in an improved survival of the graft and also in less degenerative changes observed by scanning electron microscopic examination[74].

1.6 Immunosuppression.

Immunosuppressive drugs are not routinely administered to recipients of human cardiac valves, in contrast to patients who receive allogeneic bone marrow or an organ transplantation. Further, valve donor and recipient are not matched for MHC antigens. The effect of immunusuppression on histological findings of valve rejection could be compared in patients who received heart transplants (with immunosuppressive therapy) and patients who received valve allografts (no immunosuppression). Schoen et al and the group of Melo found as most striking factor that there was a marked decrease in cellularity (fibroblasts) in the valve replacement group compared to the heart transplantation group, and T-cells were especially present in valve allografts but not into the valve leaflets of heart transplant recipients[50,75-77].

Clarke et al described a possible beneficial effect of antiinflammatory agents on allograft survival in young children. These few children, who only received anti-imflammatory drugs during the early postoperative period did not require reoperation[23]. One child was treated with low-dose cyclosporin and underwent reoperation 4 months later. The explanted allograft of this child remained free from calcification in contrast to comparable explanted valves, suggesting a protecting effect of cyclosporin on allograft degeneration[21].

Cyclosporin is a highly potent immunosuppressive drug, that has thus far only been used in individual cases in the clinic for the indication of valve replacement. The general opinion of the use of this drug after valve implantation is to use it with caution because of its serious side-effects. There are no reports in the literature of controlled follow-up studies of patients with an allogeneic valve treated with cyclosporin or other immunosuppressive drugs and the effect on valve function. However, Augelli et al examined dogs receiving venous allografts with or without cyclosporin treatment. Dogs treated with cyclosporin showed improved graft survival

compared to dogs that received no immunosuppression[78]. Yankah et al treated Lewis rats which received an allogeneic valve with a short course of cyclosporin A (10 mg/kg daily for 14 days). This resulted in the prevention of early valve degeneration observed on histological examination in the cyclosporin-treated rats, compared to rats with an allograft which were not treated with cyclosporin A[79].

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Chapter 2

AIM OF THE THESIS

Rejection is a phenomenon commonly observed after allogeneic organ or bone marrow transplantation. The destruction caused by the immunologic activation may eventually lead to graft failure[1,2]. Transplantation antigens play an important role in the initiation of a donor-specific rejection process, which consists of complex interactions of immune-competent cells of the recipient with Major Histocompatibility Complex (MHC) molecules of the donor, resulting in the activation of the cellular and humoral immune response. The cellular immune response is initiated by the presentation of donor antigens by antigen-presenting cells in the graft to immune competent cells of the recipient. This can lead to the activation of T-lymphocytes of the graft acceptor. Activated T-lymphocytes bear an Interleukin-2 receptor and can produce Interleukin-1 and other lymphokines to stimulate T-cell proliferation[3-5]. In the graft, this leads to upregulation of adhesion molecules and MHC antigens, which are rapidly recognized and destroyed by allo-reactive T-lymphocytes. The antibodies formed after initiation of the humoral immune response after bone marrow or organ transplantation, can be directed against MHC antigens or blood group antigens[6,7]. In human valve allograft transplantation, the grafts are randomly allocated to the recipients. Because of the variability of the Human Leucocyte Antigen (HLA) system, it is unlikely that valve recipients are compatible with donor transplantation antigens. Many experimental animal studies already showed findings of rejection with deterioration of the valve leaflets in allogeneic transplanted valves. These data produced in animal models can not necessarily be extended to human valve transplantation. The contribution of immunological damage to the graft and subsequently valve insufficiency still remains unclear. Many authors believed that human valves only bear a low antigeneic load, but this assumption has not been scientifically tested.

In our center, histological examination of valve allografts explanted because of valve insufficiency, showed infiltrates consisting of lymphocytes and a strongly reduced amount of fibroblasts which are normally present in a heart valve leaflet. These degenerative changes are frequently observed; degeneration still remains the most frequent cause of allogeneic valve failure[8]. Therefore we hypothesized that allogeneic valves evoke a donor-specific immune response, leading to destruction of viable cells in the graft, which may eventually lead to graft failure (valve insufficiency in the clinical setting). The aim of this thesis is to investigate the cellular and humoral allo-reactive immune response induced by fresh and cryopreserved human cardiac valve allografts. Therefore, we made use of laboratory assays which were earlier used for the evaluation of the alloreactive immune response after heart transplantation. The studies concerning the cellular immune response were performed at the department of Internal Medicine I (Transplantation Laboratory), University Hospital Rotterdam-Dijkzigt. Antibody detection took place at the department Immunohematology and Blood Bank, University Medical Center Leiden.

In *chapter 3*, we describe the results of in vitro studies. The effect of fresh valve pieces on the lymphoproliferative capacity of responder lymphocytes mismatched for HLA -A, -B, and -DR with the valve donor, was tested in a lymphocyte stimulation essay. Further, we tried to culture endothelial cells from fresh valve leaflets, which were subsequently tested in a mixed cell culture for their stimulatory effect on lymphoproliferation. We also show the effect of HLA -DR matching and cryopreservation on lymphoproliferation in lymphocyte stimulation tests.

In *chapter 4*, we examine the phenotype and donor-specific cytotoxic capacity of graft infiltrating cells. If in vivo activated T-lymphocytes, baring a T-cell receptor, are present in the explanted graft, these cells can be cultured by incubation with Interleukin-2 in an ex vivo model. Clones of the cells obtained can be phenotyped and tested for allo-reactivity.

In the next chapter the allo-reactive antibody response directed against HLA class I and class II antigens in patients who received a cryopreserved valve allograft during the first year after transplantation is described.

In *chapter 6*, the results of an immunohistochemical study directed at the cellular composition of fresh and cryopreserved valve allografts, the presence of antigen presenting cells and the expression of transplantation antigens and some immunological activation markers are shown.

Finally, the results and conclusions of the previous chapters are summarized in *chapter 7*.

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Chapter 3

THE CELLULAR IMMUNE RESPONSE EVOKED BY HUMAN CARDIAC VALVE ALLOGRAFTS IN VITRO

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Ann Thorac Surg 1995;60:S131-4.
Franciska M.E. Hoekstra, Christiaan J. Knoop, Zohara Aghai, Nicolet H.P.M. Jutte, Bas Mochtar, Egbert Bos and Willem Weimar.

In: Cardiac valve allografts. Yankah AC, Yacoub MH, Hetzer R, Eds. Darmstadt: Dr Dietrich Steinkopff Verlag; New York: Springer, 1997.
Franciska M.E. Hoekstra, Christiaan J. Knoop, Egbert Bos, Nicolet H.P.M. Jutte, Claes Wassenaar and Willem Weimar.

Abstract

Both fresh and cryopreserved human cardiac valve allografts are transplanted without matching for blood group or Human Leucocyte Antigens (HLA) with their recipient, and without the usual immunosuppressive therapy administered to patients after organ transplantation. Calcification occurs in almost all transplanted valves, and in children even acute valve failure is frequently seen. We hypothesized that failure of the human valve allografts could have an immunological basis. This hypothesis was tested in a functional way by performing lymphocyte stimulation assays using fresh and cryopreserved valve pieces and endothelial cells derived from valve leaflets as stimulator. Human peripheral blood lymphocytes, both matched and mismatched for HLA antigens were used as responder cells. The tritiated thymidine incorporation into the lymphocytes was measyred after 7 days. The results were expressed as stimulation index (SI). Fresh valve pieces induced a significantly higher SI (median: 9, range 4-117), compared to the cryopreserved (median: 2, range 0-9, p=0.002, Wilcoxon). The SI was significantly reduced when lymphocytes matched for HLA -DR with the valve pieces were used (median: 1, range 0-5) versus the HLA -DR mismatched combination (median: 4, range 2-117, p=0.006, Wilcoxon). Valve leaflet derived endothelial cells were able to induce a median SI of 8 (range 3-15) when incubated with lymphocytes mismatched for HLA -A, -B and -DR. In conclusion, stimulation of immune competent cells in vitro is induced both by fresh and cryopreserved human valve pieces and by endothelial cells derived from fresh valve leaflets. The immune response can be reduced by using cryopreserved valves or by matching between valve donor and responder lymphocytes for HLA -DR. Viable endothelial cells, present on fresh but not on cryopreserved valves could play a major role in the high immunogenicity of fresh valves. The cellular allogeneic response in vitro could be an explanation for valve allograft degeneration observed in the clinic.

Introduction

Fresh human cardiac valve allografts as well as cryopreserved valves are used for the reconstruction of congenital and acquired valve diseases. The good results during the first few years[1] after transplantation of these valves without HLA-matching between donor and recipient did not induce a discussion about the effect of histoincompatibility on the functioning of these valves. Although acute valve failure is frequently encountered, especially in children, now long-term follow-up studies show that valve calcification resulting in valve insufficiency is also commonly seen[2,3]. Apart from technical problems, immunological phenomena might result in valve destruction and this is even a likely explanation as valve donor and recipient differ in histocompatibilty antigens. Endothelial cells are the barrier between the allograft valve and the acceptor and these cells are known to evoke an immune reaction[4,5]. Therefore, the stimulatory capacity of endothelium derived from fresh valve allografts was tested in a mixed endothelium-lymphocyte reaction. We also investigated whether fresh and cryopreserved human cardiac valve pieces are able to evoke an immunologic reaction by testing their capacity to induce a proliferative response of immune-competent cells in vitro. As responder cells, peripheral blood lymphocytes mismatched for HLA -A, -B and -DR were used. The proliferative response of HLA -DR matched lymphocytes was also tested.

Material and methods

Valve allografts

Human pulmonary and aortic valve allografts were prepared under sterile conditions from hearts of heartbeating or non-heartbeating donors within 24 hours after death[6]. For the present study, aortic and pulmonary valve were studied that were not accepted for transplantation, mostly because of technical reasons. The valve allografts were all placed for 24 hours at 4^0C in a low

concentration antibiotic solution, containing flucytosin 0.03 mg/ml, vancomycin 0.012 mg/ml, amikacin 0.012 mg/ml, ciprofloxan 0.003 mg/ml and metronidazol 0.012 mg/ml in medium 199 (Gibco, Paisley, Scotland). Two fresh valve leaflets were prepared. The third valve leaflet connected to the arterial trunk was cryopreserved according to the standard cryopreservation protocol (-1^{0}C per minute) in medium 199 containing 10% DMSO. The cryopreserved leaflet was quickly thawed after 12-24 hours (standard thawing protocol). One fresh leaflet and a cryopreserved leaflet derived from the same valve were cut into pieces with a 2 millimeter diameter biopsy punch (Stiefel Laboratories LTD). These pieces were each placed in a well of a 96-well culture cluster (Costar, Cambridge,MA) with culture medium, consisting of 10% heat-inactivated human serum, 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin in RPMI 1640 Dutch Modification (Gibco, Paisley, Scotland).

Isolation of endothelial cells

The third leaflet was used to isolate endothelial cells according to the method of Johnson and Fass[7]. Therefore, the fresh leaflet was rinsed gently in medium 199 after the antibiotic bath, then it was placed for five minutes in a 1 mg/ml collagenase solution (Collagenase A, Boehringer, Mannheim) in HBSS (Gibco, Paisley, Scotland) at 37°C. It was rinsed gently in HBSS without calcium and magnesium (Gibco) and thereafter placed in 1 ml endothelial cell culture medium (RPMI 1640: medium 199 1:1 (Gibco) and 30% heat-inactivated human serum) with 50 μg/ml Endothelial Cell Growth Supplement (Collaborative Research Incorporated, Bedford, MA) and 50 μg/ml Heparin (Sigma, St Louis, MO). After 1 minute gentle shaking by hand, the leaflet was removed and the culture medium containing the endothelial cells was put into one well of a 24-well Primaria dish (Becton & Dickinson, Mountain View, CA). At confluence the endothelial cells were passaged after trypsinisation in trypsin EDTA (0.05% / 0.02%). Six sets of

endothelial cells were derived from six different valves. The purity of the cell lines was assessed by double staining with FITC-labelled Ulex Europaeus lectin and the monoclonal antibody EN4 (Sanbio, Uden, The Netherlands) in combination with anti-mouse kappa-PE (Becton & Dickinson) and subsequently analyzed on a FACScan flow cytometer (Becton & Dickinson).

Lymphocyte stimulation assay

Peripheral blood lymphocytes, 1.10^5 per well, matched or mismatched for HLA-DR were incubated in the presence of irradiated (30Gy) fresh or cryopreserved valve pieces or endothelial cells in a total volume of 200 μl at 37°C in a humidified atmosphere of 5% CO_2 in air. The [^{3}H]thymidine incorporation into the lymphocytes was measured after 7 days incubation. First the valve pieces were removed from the wells and then 0,5 uCi [^{3}H]thymidine was added to each well. After 8 hours incubation in a humidified atmosphere, the lymphocytes were harvested on glass fiber filters. The proliferation assay using endothelial cells as stimulator was performed as described above in the presence of 1.10^4 irradiated endothelial cells per well. For positive control third party Epstein Bar Virus transformed B-lymphoblastoid cell lines were used as stimulator while autologous irradiated lymphocytes served as negative controls. The [^{3}H]thymidine incorporation into endothelial cells was nihil. The ^{3}H thymidine incorporation was measured in the Beta Plate Counter (LKB, Sweden). The tests were performed in tenfold. Results were expressed as stimulation index (SI) : the cpm incorporated in lymphocytes incubated with valve pieces or in endothelial cells, divided by the cpm incorporated in negative controls.

Results

Significant proliferative responses were found in all instances where fresh valve pieces, cryopreserved valve pieces or endothelial cells were used as stimulator incubated with HLA -A, -B and -DR mismatched lymphocytes.

Fresh valve pieces induced a significantly higher SI compared to cryopreserved valves: median 9 (range: 4-117) for fresh versus 2 (range 0-9) for cryopreserved valves, p=0.002, Wilcoxon (figure 1).

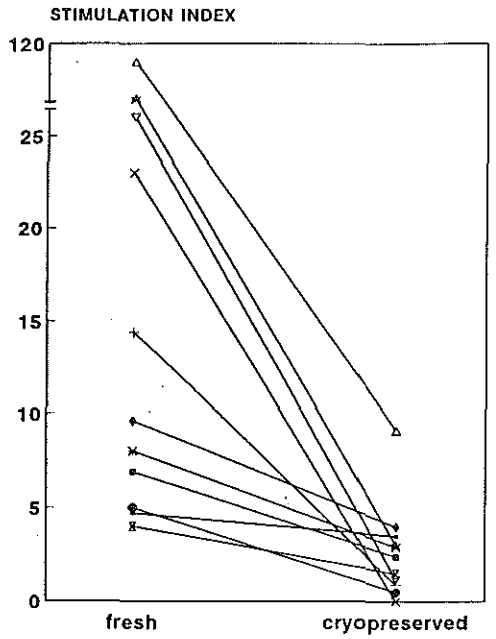

Figure 1. *Median stimulation index of lymphocytes incubated with fresh versus cryopreserved valve pieces.* All lymphocytes were mismatched for HLA-A, -B and -DR with the valve leaflets. Each line connects the median stimulation index of fresh and cryopreserved valve leaflets derived from the same valve.

Endothelial cell cultures were obtained in almost all instances (14/15) when fresh leaflets were used, while cryopreserved valves did not yield viable endothelial cell cultures in any of the 5 tested instances. Endothelial cells induced a median SI of 8, range: 3-11 (figure 2). Compared to the SI of the fresh valve pieces from the same valve, endothelial cells induced a median SI of 8 (range 3-11) versus 10 (median, range 5-35) for fresh valve pieces. In contrast, cryopreserved leaflets induced less proliferation than endothelial cells derived from the same valve, with a median SI of 2 (range 0-9).

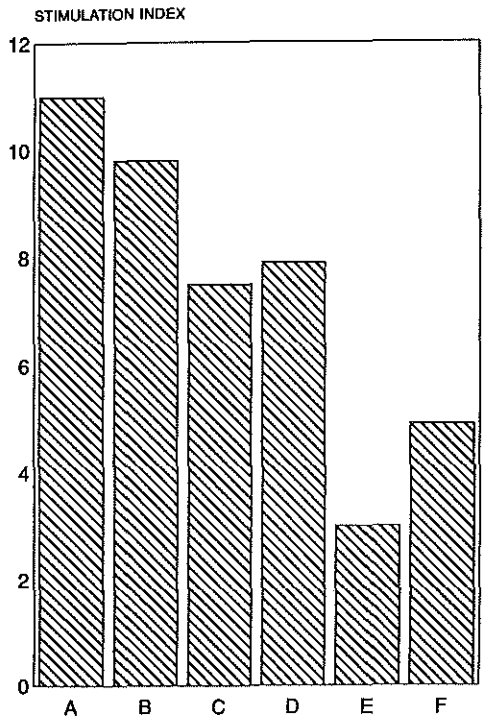

Figure 2. *Stimulation index induced by valve endothelial cells.* Bars represent different endothelial cell lines derived from six different valve leaflets.

A very weak proliferative capacity was found for HLA-DR matched lymphocytes incubated with either fresh (n=3) or cryopreserved (n=4) valves; the median SI of HLA -A, -B and -DR mismatched lymphocytes was 4 (range 2-117) versus 1 (range 0-5) for HLA-DR matched lymphocytes (p=0.006, Wilcoxon, figure 3), resulting in a median decrease of 90% due to HLA-DR matching.

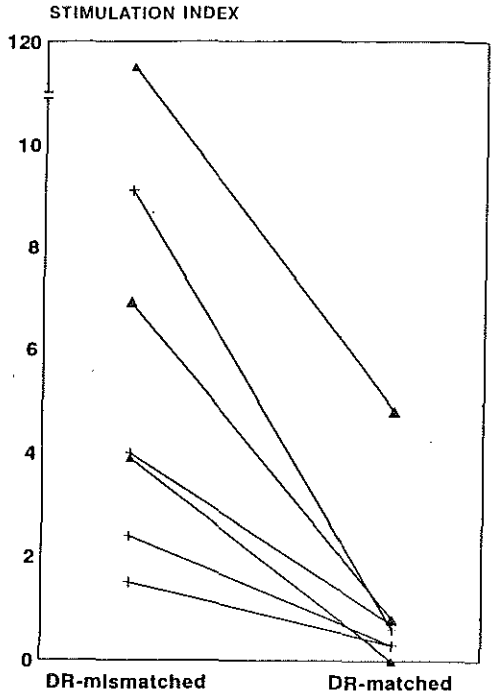

Figure 3. *Median stimulation index of lymphocytes mismatched versus matched for HLA-DR with the valve donor.* In all cases, lymphocytes were mismatched for HLA-A and -B with the valve pieces. Incubation with fresh (▲) or cryopreserved (+) valve pieces.

Discussion

Transplantation of allogeneic organs between histoincompatible individuals will give rise to an immune response. The importance of histocompatibility was earlier suggested by Yankah et al[8,9] who described infiltrates consisting of lymphocytes in transplanted allogeneic human valves. An immunologic reaction, especially the cellular reaction by human T-lymphocytes against HLA-antigens can result in destruction and dysfunction of the graft. In this study, we found that human cardiac valves evoke a proliferative response of HLA -A, -B and -DR mismatched lymphocytes, and that matching of HLA-DR between valve donor and responder lymphocytes resulted in a significantly lower stimulation of lymphoproliferation. Therefore, we suggest that an immunologic reaction is involved in the failure of human valve allografts, as

earlier described in animal studies[10,11,12,13]. In the present study we report that valve derived endothelial cells alone have the potential to activate immune competent cells. This may be the result of upregulation of HLA-antigens or other allo-antigens on their cell surface[14]. Further studies using endothelium-lymphocyte reactions could clarify the importance of HLA class I and class II. In our experiment endothelial cells could not be propagated from cryopreserved valves, suggesting the absence of viable endothelium after cryopreservation. Histological examination of cryopreserved valves in our institution did not show the presence of endothelial cells on the surface of valve leaflets. This was earlier reported by Lupinetti[15]. In parallel to these results we found a lower proliferation of immune competent cells when incubated with cryopreserved valve pieces compared to the fresh leaflets.

In conclusion, we suggest that destruction of transplanted human cardiac valves may be the result of a cellular immune response, that cryopreservation or matching for HLA -DR is associated with less immuno-stimulation and that endothelial cells present on the surface of fresh allografts could be responsible for the initiation of the immunologic reaction.

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Chapter 4

DONOR-SPECIFIC CELLULAR IMMUNE RESPONSE AGAINST HUMAN CARDIAC VALVE ALLOGRAFTS

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Franciska M.E. Hoekstra, Christiaan J. Knoop, Leonard M.B. Vaessen, Claes Wassenaar, Nicolet H.P.M. Jutte, Egbert Bos, Ad J.J.C. Bogers and Willem Weimar.

Abstract

We studied the presence of donor-specific T-lymphocytes in explanted human cardiac valve allografts in vivo. From five out of seven explants lymphocyte cultures were propagated in an Interleukin-2 conditioned medium. Phenotyping revealed the presence of T-cell receptors in more than 95% of the lymphocytes obtained in each culture. Donor-specific cytotoxicity was demonstrated in three patients with known HLA-status of the donor. In one patient, cytotoxicity was directed against HLA class I only and against class I and/or class II in the others. These results indicate that donor-specific cellular reactivity can be induced by transplantation of human cardiac valve allografts.

Introduction

Human cardiac valves are transplanted without the immunosuppressive therapy normally given to recipients of vascularized organ transplants, because it is supposed that antigenicity does not play a major role in graft survival. However, early valve failure is not uncommon, and especially in children other than technical causes are suggested to play a role[1]. In animal models the antigenicity of valve allografts was illustrated earlier[2,3]. Sensitization of the recipient against allogeneic valves was revealed by the rejection of donor specific skin transplants[4] and by the measurement of allo-antibodies[5]. Recently, a donor-specific cellular immune response in vivo has been demonstrated in rat spleen cells after valve allograft transplantation[6]. Studies on the antigenicity of human valve allografts have been limited to sparse descriptions of histological findings of infiltrates in explanted valves[7]. In addition, we showed stimulation of immune competent cells by human valve leaflets in vitro[8].

In the present study, we examined the immune response after transplantation of human cardiac valves in an ex vivo model. We cultured graft infiltrating lymphocytes from explanted human cardiac donor valves, and tested them for donor-specific reactivity.

Material and Methods

Patients.

Valve recipients were between 8 months and 60 years of age at the time of transplantation. Valve allografts (4 aortic and 3 pulmonary) were obtained from heart beating (n=6) and non-heart beating (n=1, patient 1) donors after preparation and cryopreservation according to the Standard Preparation Protocol of the Heart Valve Bank Rotterdam[9]. Three valves were placed in the aortic position and four were used to reconstruct the right ventricular outflow tract. The interval between transplantation and explantation, the

causes of valve failure leading to explantation, and HLA-type of the valve donor are shown in Table 1. Four valves were explanted between 19 and 84 months after transplantation because of structural valve failure, which refers to changes intrinsic to the valve, causing stenosis or regurgitation[10]. One valve was explanted because of nonstructural valve deterioration 6 months after transplantation. Nonstructural dysfunction refers to any abnormality resulting in stenosis or regurgitation that is not caused by intrinsic changes of the valve. Two allografts were explanted for not valve related reasons (in one case the allograft was obtained at autopsy after the recipient had died in a car accident, and in the other case it was obtained 4,5 hours after transplantation for technical reasons because of take down of intracardiac repair). HLA-typing of the valve donor was performed on spleen cells or peripheral blood mononuclear cells, isolated on Ficoll-paque (d=1.077) density gradient centrifugation (Pharmacia, Uppsala, Sweden) separation. HLA class I antigens were typed according to the standard NIH lymphocytotoxicity assay. Highly selected antisera were used for HLA-DR typing[11]. The HLA-type of the valve recipients was not available. The Medical Ethical Committee of the University Hospital Rotterdam approved the studies.

Table 1. *Patient characteristics.*

(age)	HLA-type donor	graft-survival	mode of failure
1 (25)	unknown	84 months	structural deterioration
2 (0,7)	A1A2 B7B8 DR2DR3	46 months	structural ,,
3 (35)	A2A32 B51 DR11DR13	6 months	non-structural ,,
4 (8)	A2 B62 DR2DR4	30 months	structural ,,
5 (60)	A2A30 B51B62 DR4	5 months	patient death*
6 (20)	A2 B7B60 DR2DR4	19 months	structural ,,
7 (4)	A1A32 B51B8 DR17DR12	4.5 hours	patient death*

*Patient death not related to valve allograft failure. Age: years.

Culture method

From each explanted valve, the valve leaflets were cut into 2 millimeter diameter pieces with a biopsy punch (Stiefel Laboratories LTD, UK). These pieces were placed in 96 well roundbottom tissue culture plates (Costar 3799, Cambridge, MA) with 200 μl Culture Medium (CM), which consisted of RPMI 1640 Dutch Modification (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated human serum, 10% vol/vol lectin-free Lymphocult-T-LF (Biotest GmbH, Dreiech, Germany), as exogenous source of IL-2, 4mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Irradiated (40 Gy) autologous or third party peripheral blood mononuclear cells (1.10^5 cells per well) served as feeder. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-paque. The cultures were grown at 37°C in a humidified atmosphere with 5% CO_2 and every 2 to 3 days half the medium was replaced by fresh CM. When growth of lymphocytes was observed, the pieces were removed and the contents of several wells were pooled. When sufficient cell density was reached, the contents of the wells were transferred to more wells.

Phenotypic analysis

T-cell lines were analyzed by three-color flow cytometry for the expression of cell surface antigens after staining with monoclonal antibodies. As pan T-cell marker, WT31, recognizing T-cell receptor alpha/beta (TCR alpha/beta) was used, and anti-leu3 (CD4) and anti-leu2 (CD8) were used as T-cell subset markers. Anti-leu19 (CD56) and anti-leu11 (CD16) were used as markers for natural killer (NK) cells. 11F2 was used as marker for TCR gamma/delta expression. Antibodies were directly conjugated to FITC, PE or PERCP (Becton & Dickinson, Mountain View, CA). 5.10^4 cells were incubated with fluoresceinated monoclonal antibodies for 30 minutes at room temperature. Cells were subsequently washed in PBS and 5000 cells were analyzed in a lymphocyte gate on a FACScan (Becton & Dickinson,

Mountain View, CA). Lymphocyte gate was set on Forward and Sideward light scatter, while cell debris was gated out by a threshold on Forward light scatter.

Cell-mediated cytotoxicity

A standard 4 hours ^{51}Cr release assay[12] was performed to measure the cytotoxic capacity of the cultures against a panel of target cells, sharing one or more HLA antigens with the valve donor. Three types of target cells were used: PHA-blasts as target for activity against HLA class I antigens, Epstein Bar Virus transformed B-cell lines as targets for class I and class II antigens and the K562 and DAUDI cell lines as control for NK cell and lymphokine activated killer cell (LAK) activity.

3.10^3 ^{51}Cr-labelled target cells/well were placed in 96-well round bottomed culture plates, and effector cells were added at an effector target ratio from 1:1 to 20:1. The plates were centrifuged (60g, 1 minute) and incubated for 4 hours at 37^0C in a humidified atmosphere with 5% CO_2. Supernatants were collected with a Skatron harvesting system (Skatron-AS, Lierse, Norway) and counted in a gamma counter for 3 minutes. Percentage lysis was calculated according to the formula:

$$\% \text{ lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

Maximal release was determined in four-fold from a Triton X 100 (5% v/v solution in 0.01 M TRIS-buffer) lysate of the target cells. Spontaneous release was determined in four-fold by incubation of target cells in medium (RPMI-1640 Dutch Modification, supplemented with 1% heat inactivated serum). Cultures were considered cytolytic when the experimental lysis percentage exceeded 10% at an effector:target ratio of 20:1 or greater and

the slope of the graph was positive[12].

Numerical results are presented as median with range. Clinical and allograft characteristics were compared with the Wilcoxon test or Student's t-test where approprate. A p-value smaller than 0.05 was considered to be significant.

Results

Although several explants showed macroscopic and microscopic signs of calcification, lymphocyte cultures were obtained from five out of seven available human cardiac donor valves from the valve leaflet and/or the vessel wall (Table 2). These cultures all contained 95% or more T-cell receptor bearing cells. The median percentage of CD8 positive lymphocytes in these cultures was 40% (range: 27 to 83%), while the median percentage of CD4 positive lymphocytes was 58% (range: 16 to 97%) (Table 2). One lymphocyte culture was obtained from a valve of which the HLA status of the donor was not available and could not be tested for donor specificity in a CML. One allograft had only been transplanted for 4,5 hours and served as control for the test system. This valve explant indeed did not generate a lymphocyte culture. Finally, six cultures derived from four remaining explants were tested in a CML against a panel of target cells sharing one or more HLA antigens with the valve donor. More than 60% lysis of cells sharing HLA antigens with the valve donor was observed in four cultures, derived from three explants (patient 3, 4 and 5, figure 1-4). No correlation was found between explants showing donor-specific lysis, age at time of implantation, graft survival or mode of failure. In patient 2 the culture consisted of CD4 positive cells only and showed no cytotoxic reactivity. As shown in Table 2, in two cases lymphocyte cultures were generated from both valve leaflet and vessel wall (patient 3 and 4). The phenotype of the two cultures derived from the allograft and vessel wall in patient 3 were identical, while the

phenotype of these two cultures differed in patient 4.

As negative controls, third party cells (sharing no HLA-antigens with the donor) were used in the cytotoxicity test. These cells were not lysed and in addition both NK and LAK activity also remained within relatively low ranges (figures 1-4). According to these results, cytotoxicity of the recipient lymphocytes was documented against HLA class I antigens of the donor in one patient (patient 4), while in the other two patients cytotoxicity might be directed against HLA class I and/or class II antigens.

Table 2. *Lymphocyte culture characteristics.*

	Cell growth		Phenotype cultures		Donor-specific lysis	
pat	leaflet	vessel	leaflet	vessel	leaflet	vessel
1	+	ND	52% WT31/CD4 46% WT31/CD8		ND	ND
2	ND	+		97%WT31/CD4		-
3	+	+	58% WT31/CD4 40% WT31/CD8	58%WT31/CD4 39%WT31/CD8	+	+
4	+	+	95% WT31/CD4	57% WT31/CD4 27% WT31/CD8 15% TCR gamma/delta	-	+
5	+	ND	16% WT31/CD4 83% WT31/CD8		+	-
6	-	ND				
7	-	ND				

ND: not done. TCR: T-cell receptor. pat: patient.

Figures 1-4. *Donor-specific lympholysis by valve allograft infiltrating cells.*

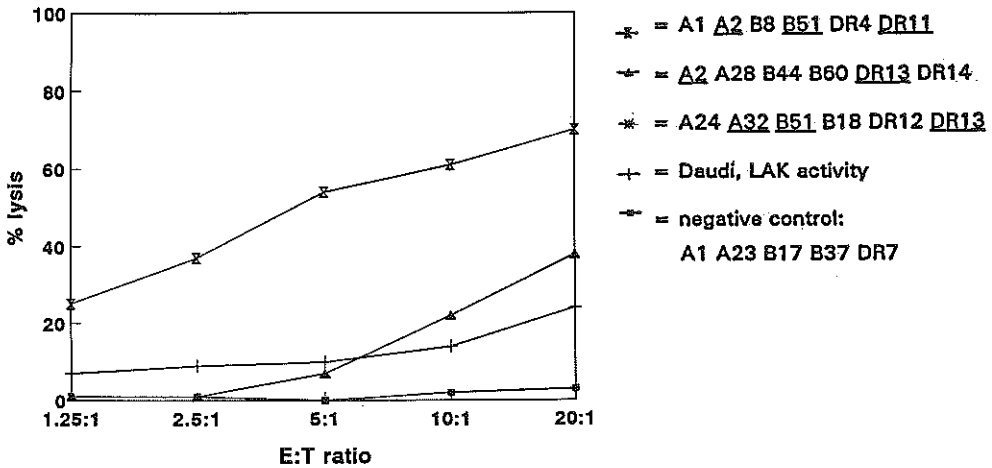

Figure 1. *Patient 3.* Lymphocyte cultures derived from the vessel wall. HLA antigens that are matched with valve donor antigens are underlined in the key. LAK: lymphokine-activated killer cell.

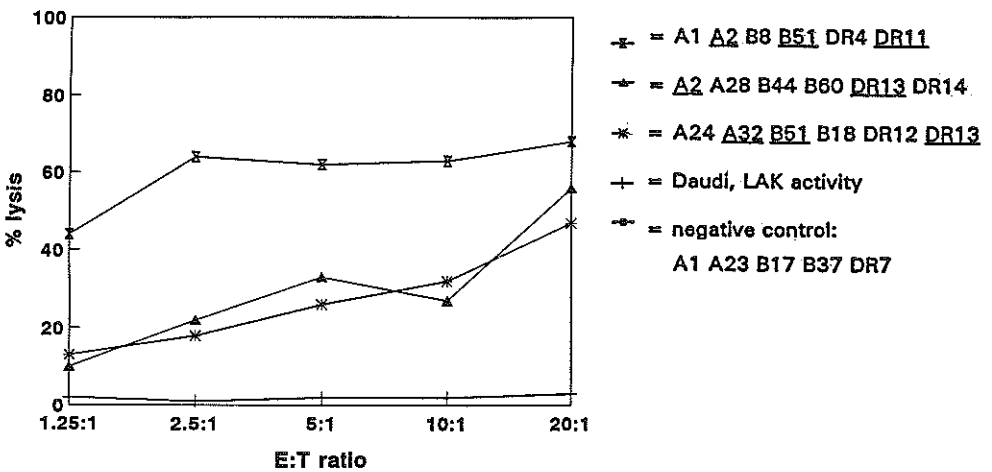

Figure 2. *Patient 3.* Lymphocyte cultures derived from the valve leaflet. HLA- antigens that are matched with valve donor antigens are underlined in the key. LAK: lymphokine-activated killer cell.

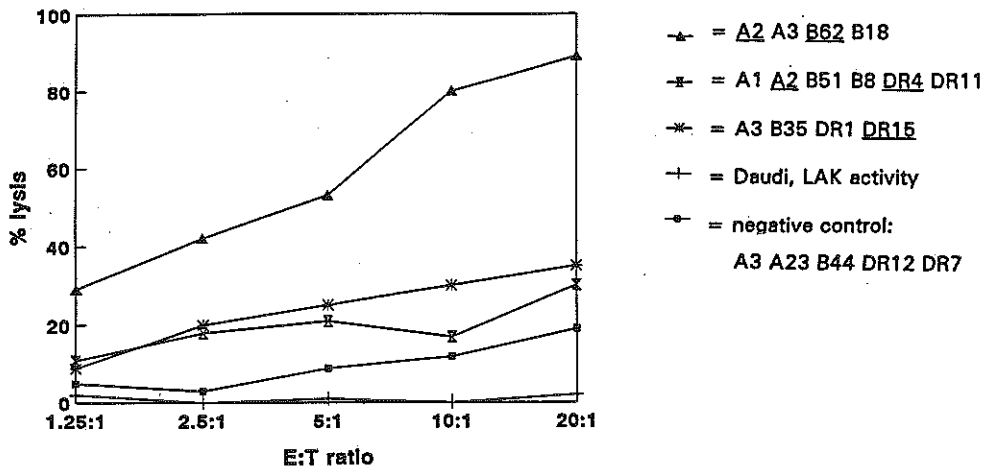

Figure 3. *Patient 4.* Lymphocyte cultures derived from the vessel wall. HLA-antigens that are matched with valve donor antigens are underlined in the key. LAK: lymphokine-activated killer cell.

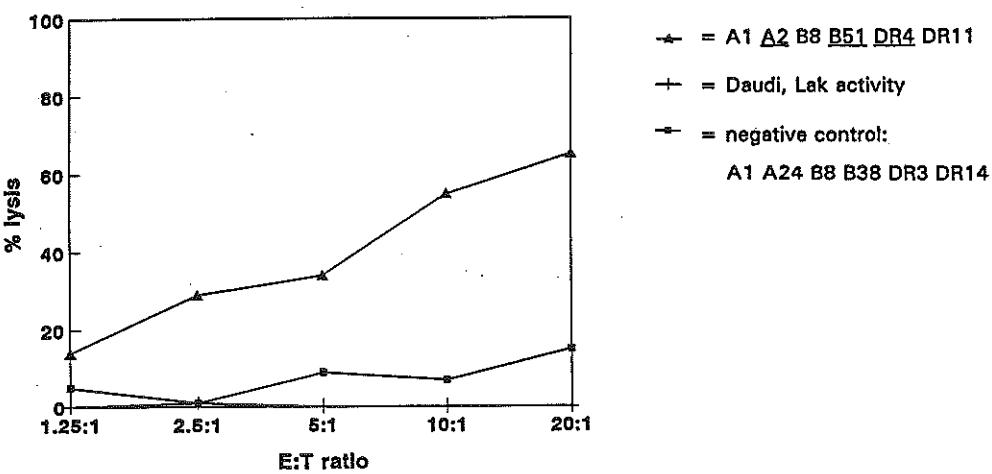

Figure 4. *Patient 5.* Lymphocyte cultures derived from the valve leaflet. HLA-antigens that are matched with valve donor antigens are underlined in the key. LAK: lymphokine-activated killer cell.

Discussion

From earlier in vitro and experimental animal studies it was clear that cardiac valve allografts are able to stimulate the immune system[2-6]. In the present study, we showed for the first time in a functional way that after valve allograft transplantation, cytotoxic lymphocytes are generated in at least some allografts (5 out of 6 in our study) that are specifically directed against donor antigens. We cultured alloreactive T-cells in IL-2 conditioned medium, in which only activated IL-2 receptor bearing lymphocytes are supposed to grow. Similar immunological methods have already been applied to characterize graft infiltrating cells after solid organ transplantation[13-15]. In these reports a correlation between the presence of donor-specific cytotoxic T-cells in the graft and rejection was described, confirming the clinical relevance of these studies.

The small number of our present series cardiac valve allografts prohibits us to make a correlation between in vivo data and patient characteristics. Nevertheless, our study unequivocally demonstrates lymphocytes infiltrating the transplanted valve, which may lead to immunological inflammation, potentially resulting in tissue damage and thus allograft dysfunction. Endothelial cells probably play a key role in this proces. Previously we showed that valve endothelial cells are able to induce lymphoproliferation, while heart endothelial cells can also be targets for cytotoxic lymphocytes[16,17]. Although valve allograft dysfunction not necessarily results in cardiac failure, a significant number of valve replacements is necessary, especially in young recipients[1].

In conclusion, our study shows that donor directed immune mechanisms are among the factors possibly leading to tissue damage and valve allograft failure. Prospective immunologic monitoring is needed to assess the relative importance of this particular factor. Manipulation of the allograft to reduce the antigenicity should be considered, and indeed, cryopreservation is associated with a reduced immunogenicity compared to fresh valve

allografts[8]. In the present study we found activity against HLA class I and class II antigens, and we already showed in vitro evidence for the importance of HLA class II matching[8] which could be considered in individual patients in case of second valve replacements and in children with their high risk of valve failure. Alternatively, low-dose immunosuppression could be given to high risk patients[1], but only for a short while because complications of long-term immunosuppression may not outweigh its benefits at the moment.

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Chapter 5

DONOR-SPECIFIC ANTI-HUMAN LEUCOCYTE ANTIGEN CLASS I AND CLASS II ANTIBODIES AFTER IMPLANTATION OF CARDIAC VALVE ALLOGRAFTS

This chapter is based on the articles published in:

J Heart Lung Transplant 1997;16:570-2.
Franciska M.E. Hoekstra, Marian Witvliet, Christiaan J. Knoop, Claes Wassenaar, Ad J.J.C. Bogers, Willem Weimar and Frans H.J. Claas.

Ann Thorac Surg, in press.
Franciska M.E. Hoekstra, Marian Witvliet, Christiaan J. Knoop, Claes Wassenaar, Ad J.J.C. Bogers, Willem Weimar and Frans H.J. Claas.

Abstract

Background. The kinetics of Panel Reactive Antibodies (PRA) and incidence of antibodies directed against Human Leucocyte Antigen (HLA) class I and class II were studied in patients who received a cryopreserved cardiac valve allograft.

Methods. A complement-dependent microlymphocytotoxicity test was used to determine the percentage PRA and the specificity of the anti-HLA class I antibodies. Anti-HLA class II antibodies were measured by two color fluorescence assays.

Results. The PRA became positive in 25/32 (78%) of the recipients between 1 and 16 months after implantation. In blood samples taken on the day of implantation, no antibodies could be detected. In 26 patients the HLA-type of the valve donor was available. In 21/23 patients of which the donor HLA-type was available and with a follow-up period of more than one month, antibodies were present. In 17 of these 21 patients (81%) antibodies were specifically directed against HLA class I of the donor. Antibodies against HLA class II antigens were detected in 11/30 (37%) patients. In 9/11 (82%) cases these antibodies were donor-specific. The induction of antibodies against donor HLA class II antigens suggests that intact HLA class II antigens are expressed by viable cells within the graft. Dithiothreitol (DTT) analysis showed that the antibodies were of the Immunoglobulin G (IgG) type. Apparently, the HLA class II antigens are expressed in an immunogeneic way as activation of specific T-Helper cells is essential for the switch from IgM to IgG antibodies.

Conclusions. Allogeneic valve transplantation is associated with the production of donor-specific anti-HLA class I and class II antibodies which could contribute to graft failure. This possibly detrimental effect might be prevented by cross matching in sensitized patients.

Introduction

Human cardiac valve allografts have been used for years for the reconstruction of cardiovascular anomalies. In adult recipients good clinical results have been reported in a number of series[1,2], although the durability of the grafts is limited. In young recipients early valve failure is more frequent[3,4,5]. In our center, the 10-years graft-survival of allografts implanted into the right ventricular outflow tract was 70% at a mean patient age of 22 years[6]. Although valve failure is related to different extrinsic factors including sizing, surgical technics and preservation methods, structural valve failure, due to intrinsic abnormalities of the valve leaflets[7] may have an immunologic basis. Allo-antigens, which stimulate the immune system of the recipient and are responsible for rejection in organ transplantations could also be present on valve allografts. Indeed, after preservation procedures like cryopreservation or fresh storage, valve allografts generally contain viable cells[8,9] capable of expressing HLA class I and class II antigens[10,11]. While the leaflet matrix is important for the long-term function of the valve leaflets, and fibroblasts are responsible for the maintenance of this matrix[12], these viable cells could be responsible for the initiation of the activation of the immune system of valve recipients. In animal studies the antigenic properties of allogeneic valves have been clearly demonstrated[13]. The immunogenicity of fresh and cryopreserved human valves in vitro is described in Chapter 3 of this thesis. High proliferative responses were found in lymphocyte stimulation assays when responder lymphocytes were mismatched for HLA -A, -B and -DR with the valve donors[14]. Endothelial cells cultured from fresh valves also induced significant proliferation of mismatched responder lymphocytes[15]. In Chapter 4 we showed that graft-infiltrating cells could be cultured from explanted allografts and their cytotoxicity was shown to be donor-specific in a cell-mediated lympholysis assay[16]. The cultures obtained were cytotoxic against donor HLA class I and/or class II antigens in 5/6 explants[16]. These data show that the cellular immune system is activated by

allogeneic valve transplantation. Antibodies directed against donor Major Histocompatibility Complex (MHC) antigens have been detected on explanted valve allografts[17]. Because of the presence of HLA class II antigens on cryopreserved valves and the cytotoxic activity of graft-infiltrating cells against these antigens, we supposed that a humoral response against HLA class II antigens could also be present. Hogan et al detected anti-HLA class II antibodies in 4/11 patients who received a cryopreserved valve allograft[18]. However, cardiac valve preparation methods vary in surgical centers, for example different concentrations of antibiotics in the nutrient medium, duration of the antibiotic bath, and varying ischemia times, which could lead to a different antigenic load of the valves before implantation. We studied the incidence of anti-HLA class I and class II antibodies in peripheral blood samples of valve recipients in our center during the first year after transplantation. We also established the kinetics of the formation of panel reactive antibodies (PRA).

Material and method

Patients

Heparin-treated blood samples were taken at varying times after implantation from 32 patients (21 men and 11 woman) who received a cryopreserved pulmonary or aortic valve allograft. The allografts had been implanted for various indications including endocarditis and congenital anomalies. The median age of the recipients was 41 years (range: 3 to 69 years). Two patients received a second allogeneic valve. Five patients were treated with steroids perioperatively e.g. because of chronic obstructive pulmonary disease and sarcoidose. The Medical Ethical Committee of the University Hospital Rotterdam and the Medical Faculty of the Erasmus University Rotterdam had approved the study and patients gave informed consent before participation.

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 standard operation procedures. Subsequently the grafts were sterilized for 24 hours at 4°C in Medium 199 (Bio-Whittaker, Alkmaar, The Netherlands) containing a low concentration antibiotic solution: vancomycin 0.012 mg/ml, flucytosin 0.03 mg/ml, amikacin 0.012 mg/ml, metronidazol 0.012 mg/ml and ciprofloxacin 0.003 mg/ml. Thereafter they were cryopreserved according to a standard cryopreservation protocol (-1°C per minute) in Medium 199 containing 10% dimethyl sulfoxide (DMSO), and stored in the vapour phase of liquid nitrogen (-160 to -180°C) at the Heart Valve Bank in Rotterdam, The Netherlands. Just before implantation, the selected valves were thawed in a 37°C bath the DMSO was removed by stepwise dilution in Medium 199.

Blood sampling

Blood samples were taken once after transplantation in 18/32 patients. From 14/32 patients 2 or more samples were collected, one sample immediately before valve implantation (negative controls) and thereafter between 1 week and 3 months, 3 to 6 months, 6 to 12 months or longer than one year after implantation.

Antibody screening

PRA and Anti-HLA class I antibody screening

Screening of patient plasma was performed by the standard National Institutes of Health (NIH) complement-dependent microlymphocytotoxicity test (LCT) against a panel of 50 selected donors carrying the majority of the serologically defined HLA-A and HLA-B specificities[19]. A positive reaction was defined as greater than 30% dead cells per well. Results were expressed as percentage panel reactive antibodies (PRA): the number of

positive donors divided by the number of donors tested. The screening was considered positive when the PRA was equal to or more than 9%.

Anti-HLA class II antibody screening

Platelet absorbed patient sera were tested for HLA class II specific antibody reactivity in a two color fluorescence assay[20]. The specificity of the antibodies was determined on the basis of their reactivity against a panel of 60 HLA-DR and HLA-DQ typed donors.

Dithiothreitol (DTT) analysis

DTT was used for the immunoglobulin (Ig) class determination[21]. A negative antibody screening test after DTT reduction is indicative for the presence of IgM antibodies only, and the detection of antibodies despite DTT treatment is an indication for IgG antibodies (with or without IgM antibodies).

Results

Incidence of panel reactive antibodies.

Panel reactive antibodies (PRA) against HLA class I were detected in 25/32 (78%) of the recipients. Eight patient samples showed no significant PRA, but in 5 out of these patients the samples were taken within 4 weeks after implantation, which could be too early to detect antibodies. Antibodies against HLA class II antigens were detected in 11/30 (37%) valve recipients. Determination of the antibodies in the presence of DTT showed that these antibodies were IgG antibodies in all cases.

Kinetic aspects.

The follow-up group consisted of 10 men and 4 woman. Five patients received more than one blood transfusion. Four patients had received an aortic allograft and the remaining patients received a pulmonary graft. In the

14 blood samples taken directly before implantation, no antibodies could be detected. The production of antibodies started between 1 to 3 months after transplantation in 6 patients and in 4 patients antibodies were present between 3 to 6 months after transplantation, but from these patients no blood sample was available for the period 0 to 3 months. The PRA remained positive for more than 6 months in 8/14 recipients (figure 1).

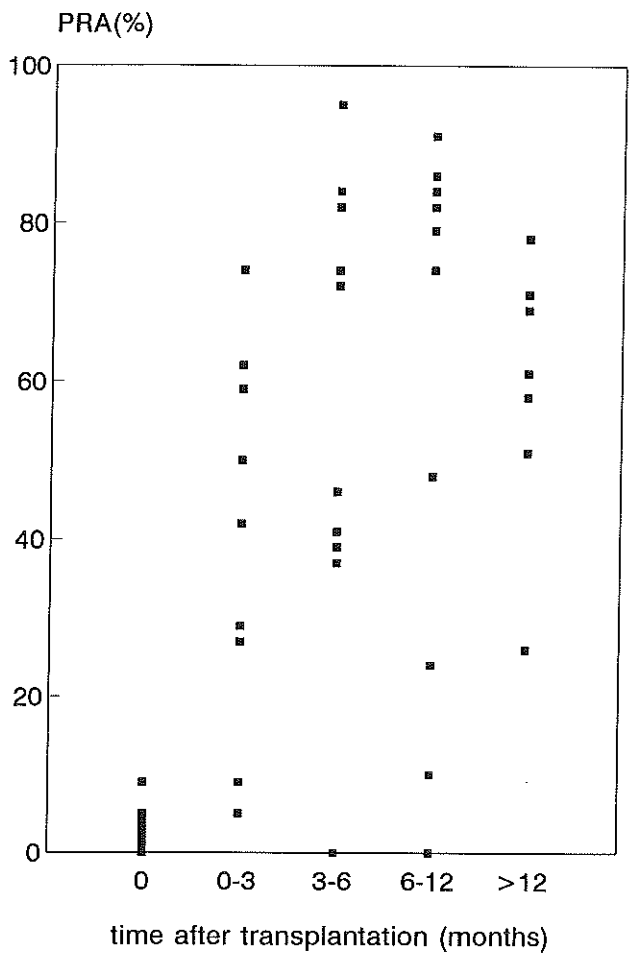

Figure 1. *Follow-up of the Panel Reactive Antibodies.* The PRA of both the cross sectional group and the follow-up group are used for this figure.

Specificity of the antibodies.

The HLA-type of the donor was available in 26 patients. In 3 of them bloodsamples were taken within the first month after transplantation. In 21/23 remaining patients (91%) with a known HLA-type of the valve donor, anti-HLA class I antibodies were detected. Out of these 21 patients, 17 patients (81%) had specific antibodies against HLA class I of the valve donor (table 1). No correlation could be found between the presence of panel reactive antibodies and perioperative blood transfusions. 6/17 patients with donor-specific allo-antibodies against HLA class I were woman, of which one had been pregnant. The presence of anti-HLA class II antibodies was measured in 30 patients. In 11/30 (37%) patients antibodies against HLA class II could be detected. Of one patient with anti-HLA class II antibodies, the HLA class II-type of the donor was not available. In 9 (82%) patients the antibodies against HLA class II were donor-specific (table 1). All patients with antibodies against HLA class II antigens had also antibodies against HLA class I antigens.

Table 1. *Specificity of the antibodies of patients of which the HLA-type of the valve donor was available and with a follow-up period of more than one month.*

Patient (sex/age)	time after Tx	BT	PRA(%)	Anti-HLA class I ab	Anti-HLA class II ab
1(f/29)	13	-	50	B7 B27	DR3
2(m/57)	13	-	27	A9	
3(f/64)	13	+	59	A2	
4(m/64)	11	+	0		
5(f/26)	12	-	77	A19	
6(m/37)	11	+	73	B7	
7(m/60)	7	-	77	A2 B7	DR1
8(m/59)	8,5	+	50	Bw4	DR7
9(m/28)	4	-	0		
10(m/26)	4,5	-	38	A11 B7	
11(f/64)	7	-	86	A1 A3 B7	DR8
12(m/65)	1,5	+	73	Bw4	DQ3
13(m/31)	3	-	73	A2 B12 B40	DR51
14(m/51)	4,5	-	40	A9	
15(m/49)	4	-	27	B17	DR8 DR52 DQ2
16(m/42)	13	+	73	A2 A9 A11	DR6(14)
17(m/26)	6	-	82	A1 A2 B40	
18(f/39)	13	-	59	A2	DR52
19(f/66)	13	+	60	B12(44) B13 B40	
20(m/57)	5	-	57	A1 A2 A11	
21(m/28)	4,5	-	40	B12 B13	
22(f/41)	6	-	50	A3 B12	
23(m/3)	9	+	91	A1 A24	DR53

Donor-specific antibodies are underlined. Tx, transplantation; PRA, panel reactive antibodies; f,female; m, male; ab, antibodies; age, expressed in years at time of transplantation.

Discussion

The implantation of cardiac valve allografts was found to be associated with the production of donor-specific anti-HLA class I and anti-HLA class II antibodies in the majority of the recipients. These results support the assumption that cryopreserved human cardiac valves contain viable cells which are able to express HLA class I and class II antigens. The immunogenicity of these antigens is reflected by the high PRA detected and the specificity of these antibodies for donor allo-antigens. Antibody production in valve recipients started approximately 4 weeks after transplantation. In patients who received blood transfusions, antibodies are generally detected already after 2 weeks after administration. The delay of antibody production in valve transplantation could be caused by a later onset of the presentation of HLA class I and class II antigens, possibly because of a recovery phase of viable cells after thawing and implantation. As compared to the humoral response after the implantation of homovital valves, antibiotic sterilization for 24 hours and the cryopreservation procedure in our center did not lead to lower percentages panel reactive antibodies after 3 months to 1 year after transplantation.

The finding that the antibodies were IgG antibodies suggests the involvement of T-Helper lymphocytes in the immune response, because these cells are necessary for the switch of the production of IgM antibodies to IgG antibodies. This activation of T-Helper cells is in agreement with earlier results of the detection of donor specific cytotoxic T-lymphocytes into explanted valve allografts, which also implies a role of T-Helper cells[16]. For the initiation of an antibody-response directed against class II antigens, direct presentation of intact donor class II antigens is necessary. Presentation of HLA class II antigens into valve allografts could be attributed to dendritic cells, endothelial cells and fibroblasts. The presence of dendritic cells in the valve leaflet has not been confirmed in the literature, but Bobryshev et al reported dendritic cells in aortic vessel walls[22]. The vessel

wall represents an important component of the valve allograft. Endothelial cells are not consistently present on cryopreserved valves. Lupinetti et al showed that cryopreservation of human donor valves is associated with loss of endothelial cells[23]. In our center histological examination showed that only a few endothelial cells were present on cryopreserved valves before implantation (unpublished data). Viable fibroblasts are found in the majority of cryopreserved valve leaflets before implantation[23], but it is not clear how long these cells survive after transplantation. Neves et al detected fibroblasts in explanted valve allografts on histological examination[24]. They also compared the number of fibroblasts in heart valve recipients and patients with a donor heart. In the heart transplantation group, which is treated with immunosuppressive drugs, the number of fibroblasts was normal. In contrast, the valve recipients who do not receive immunosuppression, the number of fibroblasts was strongly reduced. The reduction of the amount of fibroblasts in transplanted valve allografts could be caused by the cryopreservation procedure, but could also be due to immunological destruction[24]. Schoen et al described 20 explanted cryopreserved valve allografts, which were morphologically non-viable and unlikely to have active metabolic functions[25].

In conclusion, these data show that viable cells capable of the expression of HLA class I and class II antigens are present in cryopreserved human valve allografts. Allo-antibodies induced by the graft could damage transplanted tissue and therefore may contribute to graft dysfunction. We recommend to perform prospective cross matching to prevent acute antibody-mediated rejections in high-risk patients: i.e. young recipients, in need of a retransplantation.

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Chapter 6

THE CELLULAR BASIS OF THE DONOR-SPECIFIC IMMUNE RESPONSE AGAINST HUMAN HEART VALVE ALLOGRAFTS AND THE PRESENCE OF MASKED ACTIVATED ENDOTHELIAL CELLS

Submitted.

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Abstract

Objective: In vitro and in vivo studies on both animals and humans have shown that cardiac valve allografts evoke donor-specific humoral and cellular immune responses which may contribute to valve dysfunction. Therefore, we examined the cells within the valves, which might be responsible for the initiation of these immune responses.

Methods: Human valves were analysed by immunohistochemical techniques using monoclonal antibodies against cell surface determinants. From each valve, fresh and cryopreserved leaflets and vascular walls were studied.

Results: No B-lymphocytes (CD20) and hardly any CD56 positive Natural Killer cells were found. A scattered pattern of macrophages was seen throughout the leaflet and the vascular wall. Many S-100 positive cells of neural origin were found in the valve leaflets. However, no double staining S-100/CD45 antigen presenting cells were present. Although no CD31 positive endothelial cell lining was present on the valve surface, ICAM-1 and HLA class II expressing endothelial cells were detected in the vasa vasorum. Groups of CD3 positive T-lymphocytes were found on the ventricular part of the leaflets and in the tunica adventitia. No differences were observed between fresh and cryopreserved valves in the presence or the distribution of the cell types investigated.

Conclusion: These data indicate that fresh and cryopreserved valves contain activated ICAM-1 positive endothelial cells, which also express HLA class II and thus may serve as targets for a donor-specific immune responses. Moreover, these cells could also be responsible for the initiation of the immune response because of their non-professional antigen presenting properties.

Introduction

For the reconstruction of ventricular outflow tracts, cryopreserved human cardiac valve allografts have been used with good clinical results[1-3]. In our centre the overall 5 years graft-survival is 78% in patients with an allograft implanted into the right ventricular outflow tract[4]. Although degenerative changes have been observed in many explanted valve allografts, especially in young recipients[5,6,7], the relation between the degenerative findings and clinical valve dysfunction is not always clear. Important factors contributing to valve failure are donor procurement, preservation methods and surgical techniques. The antigenicity of valve allografts has been frequently reported in animal studies[8,9,10]. In previous studies we showed the immunogenicity of fresh and cryopreserved valves *in vitro*[11] and *in vivo*[12]. Hogan et al showed that cryopreserved human aortic valve allografts were able to elicit an donor-specific antibody response against HLA-antigens[14]. They also detected a significant increase in the proliferative response of recipient lymphocytes after stimulation with donor mononuclear cells on day 30 after implantation. We were able to confirm their data in a longitudinal study in patients who received a cryopreserved allograft, demonstrating antibodies directed against donor HLA class I and class II in peripheral blood[15,16]. These results of immune reactivity against transplantation antigens after implantation of a cryopreserved valve allograft suggest the presence of viable cells in the graft capable of expressing HLA class I and II antigens. The presence of HLA class I and class II positive cells have already been described by Yacoub et al in fresh human aortic allografts[17].

In the present study we examined human valves for the distribution of various cell types, including antigen-presenting dendritic cells, which are involved in the initiation of the allogeneic response. Bobryshev et al described the presence of dendritic cells in the arterial intima and in atherosclerotic lesions[18]. These dendritic cells are generally seen as the most potent antigen presenting cells. The presence and activation status of

endothelial cells on the surface and within the valve allograft was also investigated. Endothelial cells have the capacity to express HLA class II and other activation markers or adhesion molecules, making prime targets for immune reactions. However, they may also have antigen presenting capacities and thus are able to function as initiators of the immune response[19].

Because processing steps like antibiotic sterilization and the cryopreservation procedure might influence the cellular composition, we compared fresh and cryopreserved pieces of leaflets and vascular wall of the same valve allograft.

Material and methods

Valve allografts.

Two aortic and two pulmonary valves were studied derived from 3 donors, two heart beating and one non-heart beating donor. From each valve, one leaflet and the connecting piece of vascular wall were fixed in 4% formaldehyde. The remaining two leaflets and vessel roots were sterilized for 24 hours at 4^0C in Medium 199(Bio-Whittaker, Alkmaar, The Netherlands) containing a low concentration antibiotic solution: vancomycin 0.012 mg/ml, flucytosin 0.03 mg/ml, amikacin 0.012 mg/ml, ciprofloxacin 0.003 mg/ml and metrenidazol 0.012 mg/ml. Thereafter they were cryopreserved (-1^0C per minute) in Medium 199 containing 10% dimethyl sulfoxide (DMSO)[20] and stored in the vapour phase of liquid nitrogen (-160 to -180^0C) at the Heart Valve Bank in Rotterdam, The Netherlands. After 4 to 8 days the valves were thawed in a 37^0C water-bath and the DMSO was removed by stepwise dilution in 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. The samples were further processed by standard formalin fixation and

paraffin embedding procedures.

ABC immunoperoxidase procedure.

After embedding, 4μm sections were cut. The thin sections were deparaffinized and the endogenous peroxidase was blocked. If necessary antigens were retrieved by enzymatic digestion or microwave treatment. For enzymatic digestion the sections were treated with 0.1% pronase in phosphate buffered saline (PBS) at 37^{0}C for 10 minutes. To stop enzymatic treatment the sections were washed in PBS at 4^{0}C for 10 minutes. For the microwave treatment 10mM citric acid buffer(pH 6,0) was warmed till 100^{0}C. The sections were placed in the pre-heated citric acid and placed in the microwave and were treated at 100^{0}C for 10 min. Thereafter the sections were cooled until room temperature was reached. Subsequently they were washed with H_2O. After these procedures all the sections were washed in PBS. The non-specific binding was inhibited by incubation with 10% normal goat serum (DAKO, Glostrup, Denmark) in PBS with 5% bovine serum albumin (PBS/BSA) for 15 min. Thereafter, the sections were incubated with the specific antibodies listed in table 1, for 30 min or overnight at 4^{0}C for the sections of which the antigens were retrieved by microwave heating. Antibodies were diluted in PBS/BSA, in previously determined optimum concentration. After two times washing in PBS with 0.1% Tween 20, the sections were incubated for 30 min with 2% biotin-labelled multilink secondary antibody (Biogenex, San Ramon, CA) with 2% normal human serum and 2% normal goat serum. Then the sections were washed two times with PBS/Tween and the 2% avidin labelled peroxidase complex (Biogenex) was added and incubated for 30 min. After washing with PBS, final staining was performed by 7 min treatment with 3,3-diaminobenzidine (DAB) and H_2O_2, resulting in a brown precipitation. The sections were counterstained with Macer's haematoxylin. All the incubations were performed at room temperature unless otherwise noted. A multi-section was

used as positive control and for negative control sections were stained omitting the specific antibodies.

Table 1. *Monoclonal antibodies used for Immunohistochemical staining.*

Monoclonal antibody	Main specificity
JC70A	CD31 endothelial cells
1H4#	CD54 (ICAM 1)
KP1	CD68 macrophages
L26	CD20 B-lymphocytes
123C3.D5#	CD56 (NCAM 1) HK cells, neuroectodermal cells
CD3 (polyclonal)	T-lymphocytes
S-100 (polyclonal)	Dendritic cells, Schwann cells and melanocytes
CR3/43	HLA-DP, -DQ, -DR
2B11	CD45 Leucocyte Common Antigen

by Neomarkers, Fremont, USA. All others by DAKO, Glostrup, Denmark.

Double staining procedure. To demonstrate that cells positive stained by S-100 were antigen presenting dendritic cells, a double staining with CD45 was performed. In short, sections were deparaffinized and the endogenous peroxidase was blocked with H_2O_2. After washing in H_2O and PBS, the non-specific staining was inhibited by incubation with 10% normal goat serum (DAKO) in PBS/BSA. Thereafter the sections were incubated for 30 min, with specific antibody direct against CD45. After washing with PBS/Tween, the sections were incubated for 30 min with 2% biotin-labelled multilink secondary antibody (Biogenex) with 2% human serum and 2% normal goat serum. Sections were washed with PBS/Tween, followed by 30 min incubation with 2% alkaline phosphatase labelled avidin complex (Biogenex)

in PBS/BSA. After washing in PBS and 0.05M Tris-buffer (pH 8.0), the colour detection was done with Fast Blue, resulting in blue stained CD45 positive cells. Hereafter, the sections were washed in Tris-buffer and PBS and incubated for 15 min with 10% normal swine serum (DAKO) in PBS/BSA for preventing non-specific binding. Subsequently, the sections were incubated for 30 min with specific S-100 antibody, followed by two washes with PBS. Sections were incubated for 30 min with 2% swine-anti-rabbit (DAKO) in PBS/BSA, rinsed twice with PBS and followed by an incubation step with 2% rabbit peroxidase-anti-peroxidase (PAP) complex (DAKO) in PBS/BSA for 30 min. After this, the sections were washed in PBS and 0.02 M acetate buffer (pH 4.6) with 0.05% Tween 20 for 30 min, respectively. Staining was detected with 3-amino-9-ethylcarbozole (AEC) and H_2O_2, followed by washing for 30 min in acetate buffer. As result all S-100 positive cells stained red. Double stained cells CD45 and S100 positive cells would be have a purple/brown staining. All incubations steps were performed at room temperature.

Results

In all positive control sections, staining was detected even when staining in the valves could not be demonstrated. In all negative sections of which the primary antibody was omitted, no staining was observed.

No difference could be found in the intensity or distribution of the labelled cells when fresh valve leaflets were compared with the cryopreserved valve leaflets from the same valve allograft.

Both fresh and cryopreserved leaflets were hardly covered with CD31 positive endothelial cells, which are normally present on the valvular and vascular wall surfaces. Only in grooves of the cusp where the surface had a ruffled appearance, some CD31 positive endothelial cells were detected. Many CD31 positive endothelial cells were found in the vasa vasorum of the

tunica adventitia, the outer layer of vascular wall. These CD31 positive endothelial cells all expressed HLA class II and the adhesion molecule CD54 (ICAM-1)(figures 1A, 1B and 1C, suppementary sheet).

CD68 positive macrophages were present scattered throughout the leaflet and the vascular wall. Furthermore, some CD68 positive macrophages were present around the vasa vasorum in the tunica adventitia.

CD20 positive B-lymphocytes could not be detected neither in fresh nor in cryopreserved valves.

A few CD56 positive NK cells were present in 2 out of 4 valves examined. These CD56 positive cells were located in the cusp on the ventricular surface and around the vasa vasorum in the tunica adventitia.

All valve allografts showed groups of CD3 positive T-lymphocytes on the ventricular side of the valve cusp and in the tunica intima (fig 2A, supplementary sheet). CD3 positive T-lymphocytes were also present in the adventitia around the vasa vasorum.

S-100 positive cells were present in large quantities within the leaflets of all valves tested. When these cells were double stained for CD45 (Common Leucocyte Antigen), none of these cells showed a double staining for both of this antibodies (figures 2B and 2C, supplementary sheet). Most of the CD45 positive cells were seen at the ventricular side of the leaflets, while S-100 positive cells were mainly found in the central layer of the valve leaflets.

HLA class II positivity was seen in the ventricular side of the valve cusps, on morphological grounds probably on activated T-lymphocytes. Also, forming a scattered pattern throughout the valve leaflets, other HLA class II cells were present. In the tunica intima and in the tunica adventitia cells positive for HLA class II were also seen. The last two staining patterns showed similarity with the CD68 staining. Finally, the endothelial cells of the vasa vasorum were positive for HLA class II, demonstrating that these endothelial cells were activated (fig 1B, supplementary sheet).

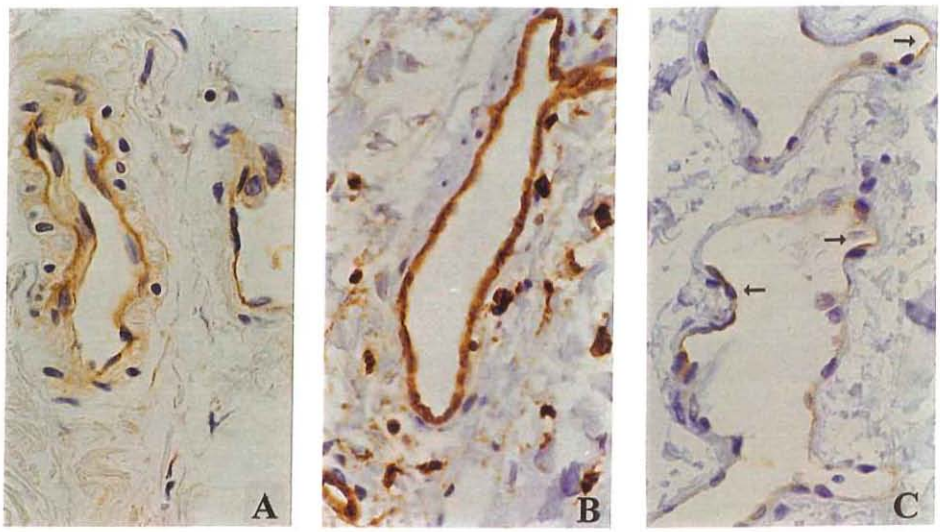

Figure 1. Immunohistochemical staining of cryopreserved human valve. **A,** CD31 staining of vasa vasorum in the tunica adventitia, showing the presence of endothelial cells. **B,** HLA class II staining of the vasa vasorum, demonstrating the presence of activated endothelial cells. **C,** CD54 (ICAM-1) staining of the vasa vasorum, the arrows show the weak, though positive staining of CD54 positive activated endothelial cells.
(All original magnifications 400x)

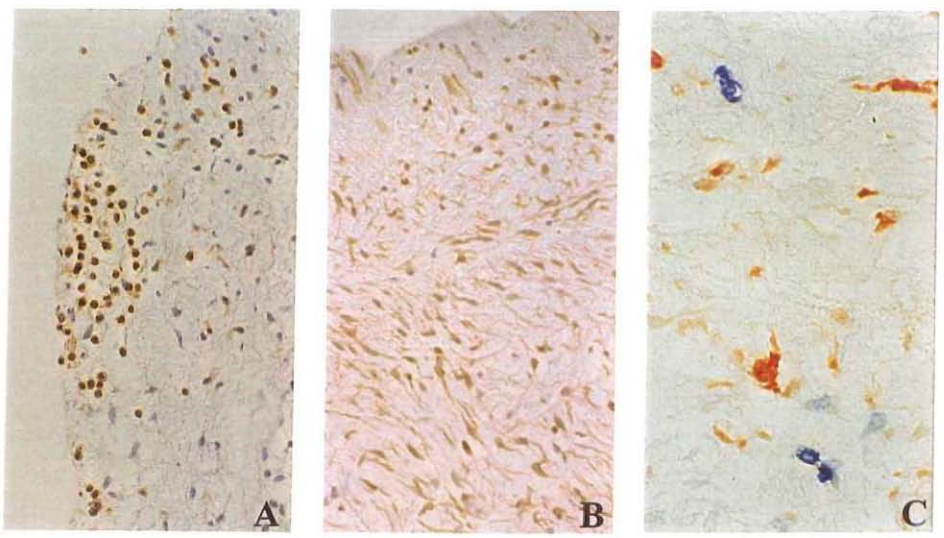

Figure 2. Immunohistochemical characterisation of immune-competent cells in cryopreserved valves. **A,** CD3 staining in the leaflet of a valve allograft, demonstrating a group of T-lymphocytes on the ventricular side of the leaflet, just below the absent endothelial lining.
B, Staining with S-100 in the valve leaflet, showing the presence of an abundance of S-100 positive cells, especially in the central area of the leaflets. **C,** Double staining for CD45 (in blue) and S-100 (in red) in the valve leaflet, demonstrating only cells in red and blue and no double stained purple/brown cells.
(Original magnification, **A** 200x, **B** 100x and **C** 400x)

Discussion

From earlier *in vivo* and *in vitro* studies it became clear that cardiac valve allografts are able to stimulate the immune system [8-16]. In the present study, we have shown immunohistochemically that both fresh and cryopreserved valve allografts contain cells capable to express class II transplantation antigens. We also found that fresh valves did not show an endothelial cell lining on the valve surface. We showed that activated endothelial cells are abundantly present in the vasa vasorum of the vascular wall, expressing ICAM-1 and HLA class II. These cells may be involved in the initial phase of the immune response, either as target or as non-professional antigen presenting cells.

Mononuclear cells from donor origin were found in all valve specimens examined, especially groups of CD3 positive T-lymphocytes in the ventricular side of the valve cusps and tunica adventitia. We did not test their viability in the present study, but our group earlier studied the survival of lymphocytes in cryopreserved endomyocardial biopsies of heart transplantation patients, and showed that T-lymphocytes indeed could be cultured from cryopreserved tissue[21].

In a morphologic study examining human valve allografts, Goffin and colleagues described the presence of S-100 positive cells in valves before and after implantation[22]. The authors reported that these cells were dendritic Langerhans cells. However, no double staining with CD45 or other controls were performed to ensure that these S-100 positive cells were of leucocyte origin. It has indeed been described that human arterial valves are heavily innervated[23]. In the present study, double stained cells were not detected, while double stained cells were present in the control sections with lymphatic tissue. Therefore, these immunohistochemical results can not confirm the presence of these professional antigen presenting dendritic cells in fresh or cryopreserved valve allografts. Nevertheless, their presence can not be excluded because these cells are scarsely present. An alterative explanation

for initiating the immune response could be the activated non-professional APC, e.g. endothelial cells or even T-cells.

In an experimental study by Mulligan et al, the effect of preservation techniques on adhesion molecules was investigated[24]. ICAM-1 was only detected in fresh valve allografts which were explanted after more than 28 days. Cryopreserved valves and valves stored at 4^0C did not express ICAM-1 even after implantation. In the present study, ICAM-1 expression in the vasa vasorum was already present at the time of arrival at the Heart Valve Bank and did not disappear after the cryopreservation procedure. The activation of the endothelial cells and consequently expression of ICAM-1 and HLA class II could have been initiated by non-specific activation caused by the brain death or perimortal stress of the donors. In a study with baboons, Novitzky et al demonstrated that in hearts after brain death, myocyte necrosis and mononuclear infiltrates are present[25]. This phenomenon could also be an explanation for the activated state of endothelium and presence of lymphocytes in the valves examined in this study.

This immunohistochemical study may give an explanation for the immune response observed *in vivo* against valve allografts. The absence of a consistent amount of antigen presenting dendritic cells and the masked position of activated endothelial cells in the vasa vasorum in the valve allograft can be an explanation for the delay in the antibody response directed against HLA class II of the donor[16]. On the other hand, the presence of activated endothelial cells, macrophages and lymphocytes of donor origin could also partially compensate for the lack of the donor antigen presenting dendritic cells.

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Chapter 7

SUMMARY AND CONCLUSIONS

Cardiac valve allografts have shown to be a good alternative for the replacement of semilunar heart valves in patients with acquired or congenital heart valve disease. The human aortic and pulmonary valve allografts have a superior hemodynamic performance, a relative resistance to endocarditis and a minimal amount of thromboembolic complications compared to porcine valve prostheses and mechanical valves. Moreover, patients with a valve allograft do not require anticoagulant therapy. Nevertheless, valve allograft degeneration is observed in allmost all explanted valves. These degenerative changes are partly caused by technical factors as surgical procedures and sizing. Further, valve insufficiency during the first year after implantation is frequently seen in young recipients. Because human cardiac valves are considered as grafts with a low antigenicity, they are transplanted without matching for ABO blood group antigens or Human Leucocyte Antigens (HLA) class I or class II between valve donor and recipient. Immunosuppressive drugs are also not routinely administered to valve recipients, in contrast to recipients of allogeneic organs or bone marrow. Therefore, an immunologic basis for valve degeneration, eventually leading to valve insufficiency is conceivable. In the present thesis we report our studies on the immunogenicity of fresh and cryopreserved human cardiac valves in vitro and in vivo.

In *chapter 1*, various aspects of cardiac valve transplantation in animals and humans are reviewed. In the introduction of this chapter, storage techniques and criteria for donation of human donor valves are briefly mentioned. Then, the results of clinical follow-up studies after implantation of human donor valves are reported. Thereafter, the results of animal studies on antigeneic properties of valve allografts are described. In these animal studies sensitization of the recipients and signs of acute and chronic rejection are reported. Further, some aspects of the immunogenicity of human cardiac valves and of

immunomodulation and immunosuppression are mentioned.

In *chapter 2* the aim of this thesis is described. In short, because valve allograft degeneration in the clinic is frequently observed, we would like to evaluate the effects of human cardiac valves on immune-competent cells in vitro and in vivo.

The results of the in vitro assays on the cellular immune response induced by human heart valve leaflets are reported in *chapter 3*. In a lymphocyte stimulation assay, both fresh and cryopreserved valve pieces stimulate the proliferation of lymphocytes mismatched for HLA -A, -B and -DR with the valve donor. The proliferative response is lower when the responder lymphocytes are matched for HLA -DR with the valve donor, compared to responder cells which are mismatched for HLA -A, -B and -DR. Cryopreserved valve pieces also induce a lower lymphoproliferation compared to fresh valve pieces derived from the same valve allograft. Endothelial cells cultured from fresh valve leaflets induce higher lymphoproliferative responses than cryopreserved valve pieces.

In *chapter 4* the presence of donor-specific cytotoxic T-lymphocytes in explanted human cardiac valves is described. The majority of the valve allografts tested were explanted because of valve insufficiency. Lymphocyte cultures could be generated in 5/7 cases after incubation in an Interleukin-2 containing culture medium, which selects only the in vivo activated lymphocytes. Phenotyping showed that T-cell receptors were present on 95% of the lymphocytes in each culture obtained. In a cell-mediated lympholysis assay, donor-specific cytotoxicity against HLA class I and/or class II antigens was detected in 3 patients of which the HLA-type of the valve donor was available.

The results of the detection of antibodies against HLA class I and class II in patients with a cryopreserved valve allograft are described in *chapter 5*. Panel Reactive Antibodies (PRA) were present in the majority of the recipients (78%) between 1-16 months after valve implantation. In the control blood samples taken on the day of transplantation, antibodies could not be detected. In 21/32 patients tested the HLA-type of the valve donor was available. In 14/17 patients

with a follow-up period of more than one month and of which the HLA-type of the valve donor was available, antibodies were present. Out of these 14 patients, 12 patients showed anti-HLA class I antibodies specifically directed against the donor. Antibodies directed against HLA class II antigens were present in 37% of the recipients examined. In 82% these antibodies were donor-specific. Dithiothreitol (DTT) analysis showed that the antibodies were of the Immunoglobulin G (IgG) type. The induction of antibodies directed against HLA class II antigens requires the presentation of intact HLA class II molecules in the graft, which can only be expressed by viable cells. The induction of IgG antibodies also confirms the immunogeneic expression of HLA class II within the valve allograft, because activation of specific T-Helper cells is essential for the switch from IgM to IgG antibodies.

The results of an immunohistochemical study on the cellular composition, the presence of endothelial cells and adhesion molecules and the expression of HLA -DR molecules in fresh and cryopreserved human valves are shown in *chapter 6*. The endothelial lining normally present on the surface of valve leaflet and vascular wall could not be detected, neither on fresh nor on cryopreserved valves. On the other hand, activated endothelial cells expressing adhesion molecules (ICAM-I) and HLA class II were present in the vasa vasorum of the tunica adventitia of fresh and cryopreserved aortic and pulmonary roots. The potent professional antigen-presenting dendritic cells could not be detected. Macrophages and lymphocytes were present in both fresh and cryopreserved valves, distributed in small groups throughout the graft. These results support the findings of previous chapters in this thesis, because activated endothelial cells, lymphocytes and macrophages of donor origin in the graft can potentially initiate a cellular and/or humoral immune response. Although immunohistochemical techniques are not developed to evaluate the viability of cells, we believe that living, activated donor cells are still present after valve transplantation. The masked position of endothelial cells and the absence of professional antigen-presenting cells could be an

explanation for the delay in the onset of the humoral immune response described in chapter 5.

We conclude that cryopreserved human cardiac valves are **not immunologically innocent** as was earlier assumed by some authors. This is illustrated by the stimulation of immune-competent cells in vitro induced by human valve pieces, the presence of donor-specific cytotoxic T-lymphocytes in explanted valve allografts, the production of antibodies specifically directed at donor antigens in the majority of the valve recipients and the immunohistochemically detected activated endothelial cells and other potential antigen-presenting cells. When these results are extended to the clinical setting, some recommandations could be done. First, to prevent hyperacute antibody-mediated rejections in sensitized recipients, we suggest crossmatching before valve implantation. Further, the HLA-type of valve donor and recipient should be available to study the long-term effect of immunohistincompatibility on graft survival. Up till now, recipients of valve allografts are not routinely HLA-typed. Matching for HLA class I and/or II between donor and recipient could be performed, although this may not be practicable because of the shortage of donor valves. In our opinion, the use of immunosuppressive drugs should be limited to high risk patients, but only for a short while and low-dose because complications of long-term immunosuppression may not outweigh its benefits.

SAMENVATTING EN CONCLUSIES

Transplantatie is inmiddels een geaccepteerde methode om zieke weefsels of organen te vervangen. Menselijke donor-hartkleppen kunnen geïmplanteerd worden ter vervanging van niet goed functionerende aorta-en pulmonaalkleppen. De transplantaatoverleving van deze humane kleppen bij oudere ontvangers is vergelijkbaar met de overlevingsduur van mechanische klepprothesen. Verder hebben menselijke hartkleptransplantaten belangrijke voordelen vergeleken met varkenskleppen en mechanische klepprothesen. Ten eerste is het niet noodzakelijk dat patiënten met een humane donorhartklep behandeld worden met bloedverdunnende medicijnen. Ten tweede zijn het de enige kleppen die gebruikt kunnen worden ter vervanging van falende hartkleppen ten gevolge van acute endocarditis (ontsteking van de hartklep). Tenslotte kan met een humane hartklep een zo fysiologisch mogelijke uitstroombaan uit de ventrikel geconstrueerd worden en is het risico op thromboembolische complicaties bij de humane kleppen het laagst. Desalniettemin ontstaan er degeneratieve afwijkingen in klepslippen en de vaatwand in bijna alle kleptransplantaten op lange termijn na implantatie. Hoewel chirurgisch-technische factoren en verschillen in grootte van de getransplanteerde hartklep ten opzichte van de vaten van de klep-ontvanger ook kunnen leiden tot degeneratie van het getransplanteerde weefsel, kunnen hierdoor lang niet alle degeneratieve verschijnselen verklaard worden. Indien er bij explantatie geen duidelijke chirurgisch-technische oorzaak voor het kleptransplantaatfalen gevonden wordt, spreekt men van **structureel** klepfalen. Bij jonge kleptransplantaat-ontvangers wordt frequent klepfalen op korte termijn (binnen een jaar na implantatie) geconstateerd, waarvoor implantatie van een nieuwe donorklep noodzakelijk is. Omdat de humane hartkleppen beschouwd worden als transplantaten met een lage antigeniciteit, worden zij geïmplanteerd

zonder te matchen voor de transplantatie-antigenen, de "Human Leucocyte Antigens " (HLA) klasse I en II tussen klepdonor en klepontvanger. Tevens worden patiënten met een humane donorhartklep niet routinematig behandeld met immunosuppressiva, zoals dit wel standaard plaatsvindt bij ontvangers van allogeen beenmerg of een orgaantransplantaat (nier, hart, lever). Een immunologische oorzaak voor de in de kleptransplantaten gevonden degeneratieve afwijkingen is daarom niet ondenkbaar. In dit proefschrift worden de resultaten beschreven enkele studies over de immunogeniciteit van verse en gecryopreserveerde menselijke donorhartkleppen.

In *hoofdstuk 1* worden verschillende aspecten van hartkleptransplantatie bij dieren en mensen samengevat, die bekend waren in de literatuur voordat aan het onderzoek beschreven in dit proefschrift werd begonnen. In de introductie van hoofdstuk 1 worden de verschillende opslagtechnieken van humane donorhartkleppen beschreven. Vervolgens worden de resultaten genoemd van klinische follow-up studies met betrekking tot de transplantaatoverleving. Daarna volgt een overzicht van de resultaten van onderzoek gericht op de antigeniciteit van hartkleptransplantaten bij dieren. Hieruit blijkt dat hartkleppen in staat zijn tot sensibilisatie van de ontvangers. In de klepslippen werden tekenen gevonden van chronische en acute afstoting. Vervolgens worden enkele aspecten genoemd van het reeds verrichte beperkte onderzoek naar de immunogeniciteit van humane donorkleppen. Tenslotte worden enkele vormen van immunomodulatie en immunosuppressie genoemd.

In *hoofdstuk 2* wordt het doel van dit proefschrift beschreven. Kort samengevat worden enkele immunologische fenomenen in vitro en in vivo getoond die op kunnen treden na implantatie van allogeen weefsel, met als doel het in de kliniek vaak voorkomende structurele kleptransplantaatfalen te kunnen verklaren.

De resultaten van in vitro onderzoek gericht op de cellulaire immuunrespons van humane hartkleppen worden getoond in *hoofdstuk 3.* Verse en gecryopreserveerde stukjes hartklep worden getest op hun capaciteit om

lymfocyten te stimuleren tot proliferatie in een lymfocyten stimulatie test. Zowel verse als gecryopreserveerde klepstukjes zijn in deze test in staat tot stimulatie van de lymfoproliferatie, wanneer de lymfocyten gemismatched zijn met de klepdonor voor HLA -A, -B en -DR. Deze proliferatieve respons is lager wanneer de lymfocyten gematched worden voor HLA -DR met de klepdonor. Gecryopreserveerde klepstukjes geven ook een lagere stimulatie van de lymfoproliferatie vergeleken met verse klepstukjes van hetzelfde transplantaat. Endotheelcellen kunnen gemakkelijk geïsoleerd en gekweekt worden van verse hartklepslippen. Deze cellen zijn in staat tot een hogere stimulatie van de lymfoproliferatie dan gecryopreserveerde hartklepstukjes. Endotheel vormt de eerste barrière tussen donor- en acceptorcellen en zou hierom een rol kunnen spelen bij de initiatie van de immuunrespons.

In *hoofdstuk 4* wordt de aanwezigheid van donor-specifieke T-lymfocyten in geëxplanteerde kleptansplantaten beschreven. Van zeven onderzochte explantaten werden er vijf verwijderd vanwege klep-insufficiëntie. Door kleine stukjes geëxplanteerde hartklep te incuberen in een kweekmedium met Interleukine 2, kunnen in vivo geactiveerde lymfocyten prolifereren, zodat lymfocytenkweken ontstaan. Uit vijf van de zeven explantaten werden lymfocytenkweken verkregen, die elk uit meer dan 95% T-lymfocyten bleken te bestaan. Drie van deze kweken, waarvan tevens de HLA-typering van de donor bekend was, bezaten donor-specifieke cytotoxiciteit tegen HLA klasse I en/of II, getest in een "cell-mediated lympholysis assay".

De resultaten van de bepalingen van antilichamen bij patiënten met een gecryopreserveerd hartkleptransplantaat worden vermeld in *hoofdstuk 5*. Panel Reactive Antibodies (PRA) werden gedetecteerd bij 78% van de onderzochte patiënten tussen een en zestien maanden na implantatie. In de controlegroep, waarbij bloedmonsters afgenomen werden op de dag van transplantatie, konden geen antilichamen aangetoond worden. De HLA-typering van de hartklepdonor was bekend van 21 van de 32 onderzochte patiënten. De follow-up periode was in 17 van deze 21 gevallen langer dan een maand. Veertien

van deze 17 patiënten hadden antilichamen gericht tegen HLA klasse I, waarvan de antichamen bij 12 patiënten specifiek gericht waren tegen HLA klasse I van de donor. 37% van de patiënten bezaten antilichamen tegen HLA klasse II, waarvan 82% donor-specifiek was. Verder liet analyse met Dithiothreitol (DTT) zien dat de gevormde antilichamen behoorden tot de Immunoglobuline G (IgG) klasse. Het feit dat er bij hartkleptransplantatie antilichamen gevormd worden tegen HLA klasse II, suggereert dat er in het transplantaat levende cellen aanwezig zijn, die intacte HLA klasse II molekulen tot expressie brengen. De vorming van IgG antilichamen bevestigt dat intacte HLA klasse II antigenen tot expressie gebracht worden, omdat voor de switch in antilichaamproductie van IgM naar IgG activatie van specifieke T-Helper cellen essentieel is.

In *hoofdstuk 6* worden de resultaten weergegeven van immunohistochemisch onderzoek gericht op de aanwezigheid van antigeenpresenterende cellen, endotheelcellen met eventuele adhesiemolekulen en de expressie van HLA-DR in verse en gecryopreserveerde humane hartkleppen. Endotheelcellen waren nauwelijks aanwezig aan de oppervlakte van de klepslippen of vaatwand bij zowel verse als gecryopreserveerde allografts. Echter, in de vasa vasorum van de adventitia van verse en gecryopreserveerde kleppen waren wel veel endotheelcellen aanwezig, die bovendien adhesiemolekulen (ICAM-I) en HLA-DR tot expressie brachten. Dendritische cellen (potente professionele antigeen presenterende cellen) werden met deze techniek niet gevonden. Vanwege de lage frequentie waarin deze cellen voorkomen kan er echter sprake zijn van een sampling-error. Verder werden enkele macrofagen gezien en tevens groepjes lymfocyten, verspreid in het transplantaat. Deze resultaten ondersteunen de bevindingen vermeld in voorgaande hoofdstukken, omdat de donor HLA klasse II antigenen, lymfocyten, macrofagen en geactiveerde endotheelcellen in het transplantaat een humorale en/of cellulaire immuunrespons tegen de donor op gang zouden kunnen brengen. Hoewel de immunohistochemische techniek geen informatie geeft over de

levensvatbaarheid van de in het transplantaat aanwezige cellen, denken wij toch dat levende cellen van donor origine aanwezig zijn tijdens en na de implantatie procedure. Dat de antilichaamrespons later optreedt dan verwacht, zou verklaard kunnen worden door het feit dat de immunogene donorcellen niet direct in contact kunnen komen met immuuncompetente cellen van de acceptor, zoals het endotheel dat "gemaskeerd" is vanwege het voorkomen in de kleine vaatjes van de adventitia.

Wij concluderen dat humane hartkleppen immunologisch niet onschuldig zijn, zoals in het verleden door meerdere auteurs werd beweerd. Dit wordt geïllustreerd door de in dit proefschrift beschreven stimulatie van immuuncompetente cellen in vitro in de aanwezigheid van verse of gecryopreserveerde klepstukjes, de aanwezigheid van specifiek tegen de donor gerichte antilichamen en cytotoxische T-cellen in geëxplanteerde kleptransplantaten en de door immunohistochemische kleuringen aangetoonde geactiveerde endotheelcellen en andere niet professionele antigeen-presenterende cellen. Naar aanleiding van bovengenoemde bevindingen zouden enkele aanbevelingen gedaan kunnen worden voor toekomstige hartkleptransplantaties. Om acute, antilichaam-gemedieerde afstotingen bij gesensibiliseerde patiënten te voorkomen adviseren wij om voor implantatie van een donorhartklep kruisproeven te verrichten. Verder zou de HLA-typering van zowel klepdonor als klepontvanger bekend moeten zijn, om het effect van histoïncompatibiliteit op de klinische functie van de hartklep op lange termijn te kunnen bestuderen (tot op heden wordt de HLA-typering van de hartklep-acceptor namelijk niet routinematig bepaald). Vanwege het gunstige effect van HLA -DR matching op de stimulatie van de lymfoproliferatie in vitro, is in de kliniek mogelijk ook een gunstig effect op de transplantaatoverleving te verwachten. Waarschijnlijk is het matchen voor HLA klasse I en/of II antigenen in de kliniek momenteel echter niet haalbaar vanwege het tekort aan donoren. De toediening van immunosuppressiva in lage dosis en voor een beperkte tijd

zou beperkt moeten blijven tot de groep patiënten met een hoog risico op transplantaatfalen, omdat de bijwerkingen van deze therapie op de lange termijn waarschijnlijk niet opwegen tegen de voordelen.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 31 maart 1968 in Rotterdam. In 1986 behaalde zij het Atheneum-B diploma aan het Jacob Roelandslyceum te Boxtel. Zij startte in hetzelfde jaar met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam, waar in 1993 het artsexamen werd verkregen (cum laude). Aansluitend werd begonnen met het in dit proefschrift beschreven onderzoek op het Transplantatie Laboratorium Interne I (Prof. Dr. W. Weimar), Academisch Ziekenhuis Rotterdam (AZR). In augustus 1994 werd op het VI International Symposium On Cardiac Bioprostheses in Vancouver, Canada, de Carpentier-Edwards Basic Research Award verkregen voor de voordracht over de immunogeniciteit van humane hartkleppen in vitro. Vanaf januari 1994 was zij werkzaam als arts-assistent niet in opleiding op de afdeling Interne I (Prof. Dr. M.A.D.H. Schalekamp) in het AZR. Aansluitend begon zij op 1 januari 1996 aan de opleiding tot internist in het Havenziekenhuis te Rotterdam (opleider: Dr. A.G.C. Bauer). Momenteel is zij werkzaam als arts-assistent op de afdeling Interne Geneeskunde I om de opleiding te voltooien.