Rejection Pathways in Heart Transplant Recipients

Afstotingsroutes in Harttransplantatie Patiënten

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Rejection Pathways in Heart Transplant Recipients

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Voor mijn mannetjes

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List of abbreviations

AR	acute rejection
B-LCL	B-cell line
CI	confidence interval
CML	cell-mediated lympholysis
cpm	counts per minute
cCTL	committed cytotoxic T-lymphocyte
CsA	cyclosporin A
CTLf	cytotoxic T-lymphocyte frequency
CTL(p)	cytotoxic T-lymphocyte (precursor)
(d)APC	(donor) antigen-presenting cell
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EMB	endomyocardial biopsy
FACScan	fluorescence activated cell scan
GIL	graft-infiltrating lymphocytes
GVD	graft vascular disease
HLA	human leukocyte antigens
HTLf	helper T-lymphocyte frequency
HTL(p)	helper T-lymphocyte (precursor)
HTx	heart transplantation
IFN	interferon
FITC	fluorescein isothiocyanate
Ig	immunoglobulin
IL	interleukin
LDA	limiting dilution analysis
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MoAb	monoclonal antibody
(m)RNA	(messenger) ribonucleic acid
NK	natural-killer
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PHA	phytohaemagglutinin
(r)APC	(recipient) antigen-presenting cell
SI	stimulation index
TCR	T-cell receptor
TET	tetanus toxoid
TGF	transforming growth factor
Th	T-helper cell
TNF	tumour necrosis factor

Chapter 1

General introduction

1.1. Human heart transplantation

Since the beginning of this century experimental heart transplantations in animal studies were performed.[1] These studies were started in Rotterdam in the seventies to compare heterotopic and orthotopic heart transplantations, and to study the process of chronic rejection.[2-4]
The history of the first human heart transplantation started in South-Africa and it was carried out by Barnard in 1967.[5] Several cardiac surgeons around the world initiated new transplantation programmes. However, the problems with patient and donor organ selection as well as with immunosuppression, severe rejection and infection were common. This meant in 1968, only 22% of all transplants survived after the first year.[6] Consequently, many centres stopped their programmes.
Heart transplantation, as a routine treatment for organ failure, only became possible with the development in 1973 of the endomyocardial biopsy technique for monitoring acute rejection[7] and in 1975 by the further discovery of cyclosporin A.[8] In the early eighties cyclosporin A was successfully introduced as an immunosuppressive medicine post clinical heart transplantation.[9] Cyclosporin A acts by binding to calmodulin and thereby inhibits the transcription of the IL-2 and IFN-γ gene.[10] With the development of these new processes, a 50% survival rate after 5 years was achieved in 1982.[11]
The first Dutch patient received a human cardiac allograft in June 1984 in Rotterdam. Up to January 1993, 200 patients with end-stage heart disease received a cardiac allotransplant with a 92% patient survival rate after 1 year, and 84% survival rate after 5 years.[12]
Because the donor heart is derived from a genetically nonidentical (non-self) donor, an immune response directed to the foreign graft will occur. Therefore, life-long immunosuppression is necessary to prevent the immune system from rejecting the allograft. Of all the foreign antigens that can be recognized by the immune system of the organ recipient the 'major histocompatibility complex' is the most important.

1.2. Organ allograft rejection

The major problem after organ transplantation is graft rejection. Allograft rejection falls into 4 categories and can be identified by the time-frame in which it develops after transplantation.

Hyperacute rejection
Hyperacute rejection occurs very rapidly within the first 24 hours after transplantation and can occur in patients with pre-existing humoral antibodies against donor cells.

Patients who already have circulating antibodies against the allograft due to ABO-incompatibility between recipient and donor, prior blood transfusions, pregnancies or previous transplants are at risk for immediate graft loss through hyperacute rejection.[13] In the graft, preformed antibodies fix complement which causes endothelial cell damage resulting in leakage of cells, fluids and platelet aggregation leading to blocking of the microvasculature by thrombosis and ultimately depriving the graft of blood.
This immediate graft loss through hyperacute rejection can be avoided by performing cross-match studies before transplantation. Serum from the patient is screened first against a panel of cells with different HLA-types to determine the level of panel reactive antibodies and then just before transplantation against donor cells for the presence of donor-specific cytotoxic anti-HLA antibodies.[14,15] Only patients without anti-donor HLA antibodies will be transplanted.
In the University Hospital Rotterdam-Dijkzigt hyperacute rejection did not occur in any of the 200 heart transplant recipients.[12]

Accelerated acute rejection
Accelerated acute rejection or delayed hyperacute rejection takes place within the first five days after transplantation. In these patients the cross-match studies are negative preoperatively. Accelerated allograft rejection, when compared to acute rejection, is a more severe rejection form with earlier onset, higher fever, and a more prolonged period of rejection.[16] This type of rejection is described as frequently reversible by some[16] and completely irreversible by others.[17] It is evident that the process of accelerated rejection has not yet been clearly defined. Whether it involves separate cellular or humoral factors, or both, remains to be elucidated.
Three out of the 200 cardiac transplant recipients from the transplant centre in Rotterdam suffered from this type of rejection resulting in graft dysfunction.[12]

Acute rejection
After the first week of transplantation, most (75%) of the heart transplant patients experienced at least one acute rejection episode.[18,19] In kidney transplant recipients the diagnosis of rejection can be made on clinical grounds (rising creatinine, oliguria, fever, and graft tenderness), and confirmed by core needle biopsy.[20] In contrast, acute rejection in heart transplant recipients can only be monitored by cardiac biopsies taken from the right ventricle via the right jugular vein. Biopsies are performed weekly during the first 6 weeks, thereafter twice monthly up to 3 months, monthly up to 6 months, every 2 months for the rest of the first year, and continuing at intervals of every 4 months thereafter.[18] Grading of rejection is histologically determined in endomyocardial biopsies (EMB) according to the criteria of Billingham et al.[21] Scoring indices register the infiltration of mononuclear cells and signs of myocyte damage or even necrosis. In severe acute rejection edema, haemorrhage

and vasculitis are usually present.
Acute rejection episodes are treated by pulsed high doses of methylprednisolone, or by polyclonal rabbit-antithymocyte globulin, and in cases of ongoing or frequently recurring rejection episodes by monoclonal antibodies against the T-cell receptor (OKT3).[19]

Chronic rejection

The long-term success of organ transplantation is limited by the development of chronic rejection. Chronic rejection is primarily an occlusive disease of the donor vessels in an allograft, which leads to ischemia and subsequently to organ destruction.
The pathology of chronic cardiac rejection may occur quite early postoperatively, often within the first 3 months and in other patients within 3 to 5 years.[22] In human cardiac allografts, it is characterized by a process of accelerated coronary artery disease, and is also called graft vascular disease (GVD). GVD can be visualized by angiography or ultrasound techniques.
In common (non-transplant) atherosclerosis eccentric narrowing of the vessels occurs, in which nonsymmetric deposits of occlusive plaque develop in the larger vessels.[23] GVD is characterized by a symmetric occlusion along the entire length of vessels.[24] This type of occlusion often continues into smaller vessels within the myocardium itself.[25] GVD seldom affects the veins of the transplanted heart; lesions are primarily restricted to the large and small coronary arteries and the aorta, which is attached to the donor heart.[22]
The exact pathogenesis of GVD is unclear. However, arterial lesions develop in the donor vessels and are absent in the recipient vessels, which suggests that GVD results from an immune-mediated process, initiated at and located within the donor vasculature.
In the transplant centre of the University Hospital Rotterdam-Dijkzigt, heart transplant recipients are routinely checked for GVD by angiography at one year after transplantation. GVD is defined as all or any vascular wall irregularities including minimal wall changes. One year after transplantation 26% of the patients transplanted in Rotterdam had signs of GVD in their angiograms, increasing to 80% 10 years after transplantation. Therefore, it can be concluded that GVD is an inevitable complication affecting almost all heart transplant recipients.
No correlation between the occurrence of GVD and a wide range of factors (such as gender, age, ischemic heart disease before transplantation, hypertension, serum cholesterol and triglycerides, use of calcium antagonists, and cytomegalovirus infection or disease) has been determined.[26] Since the vascular lesions only develop in donor vessels and remain absent in recipient vessels an allogeneic immune process is possibly involved in the pathogenesis of GVD. During the searches carried out in the transplant centre of Rotterdam, no immunological parameters (the

instances of acute rejection episodes, preoperative reactive antibodies against a panel of lymphocytes and number of HLA mismatches) were detected in relation to GVD.[26]
Currently, no treatment for chronic rejection exists and retransplantation is the only solution.

1.3. Major histocompatibility complex and T-cell receptor

Major histocompatibility complex
The genetic loci involved in rejection of an allograft form a region known as major histocompatibility complex (MHC). One way to overcome rejection of the graft is matching for these histocompatibility antigens. The human MHC, known as the human leucocyte antigen (HLA) system is a set of genes coding for highly polymorphic cell surface structures. It is located on the short arm of chromosome 6.[27]
Three sets of MHC genes are identified. The class I and class II genes are involved in triggering T-lymphocytes, while class III genes are related to the components of the alternative complement activation pathway, and are located between the HLA class I and II genes. The extreme polymorphism of the HLA-system results in a vast number of HLA-types. MHC class I genes are expressed in all nucleated cells, whereas class II genes are only found on cells which are involved in antigen presentation (dendritic cells, macrophages, monocytes, B-cells, activated T-cells and endothelial cells).
MHC class I molecules present foreign and self peptides to $CD8^+$ T-cells. The class I region encodes HLA-A, HLA-B, HLA-C and five not completely characterized loci (HLA-E, -F, -G, -H, -J).[28] Class I antigens are composed of a highly polymorphic heavy chain (α-chain) and a noncovalently associated nonpolymorphic β2-microglobulin. Epitopes involved in alloantigen recognition are located on the α-chain, which is organised in three globular domains (α1, α2, α3). The α1 and α2 domains are the most polymorphic domains of the class I gene.[29] Peptides of 8 to 10 amino-acids are bound on the groove formed by these two domains. Several polymorphic residues are located in the part of the molecule that constitute this groove, resulting in the ability to bind different peptides.
Class II genes, encoded by HLA-DR, HLA-DQ and HLA-DP, are heterodimers consisting of a heavy α-chain and a light β-chain, each encoded by a separate gene A and B, respectively. Both chains have two extracellular domains: α1 and α2, β1 and β2. Class II polymorphism is concentrated in the peptide binding groove formed by the β-chains in DR molecules and by the α1 and β1 domains in DQ and DP molecules. In contrast to class I molecules, the groove of class II molecules is open at both ends, resulting in longer peptide binding (12-25 amino-acids) that extends out

of the cleft.[30]

T-cell receptor

T-cells recognize antigens bound to MHC molecules using a specific receptor: the T-cell antigen receptor (TCR). There are two types of TCR: TCR-αβ and TCR-γδ. Approximately all T-cells express the TCR-αβ together with the CD4 or CD8 molecule, while only a few T-cells express the TCR-γδ together with the CD8 molecule. The latter type of T-cells have a non-MHC restricted repertoire,[31] while the TCR-αβ-complex recognizes antigen in association with MHC. Both types of TCR are associated with the CD3-complex.

The TCR-αβ compromises a disulphide-linked heterodimer of an α and β chain. The diversity of the T-cell repertoire is created by the rearrangement of the gene. Both chains have a constant C region. In addition, the α-chain is encoded by V (variable) and J (joining) gene segments, and the β-chain by V, D (diversity) and J segments.[32] Within the V gene there are three areas of relatively greater variability present (complementarity-determining region (CDR) 1, CDR2 and CDR3).[32] These hypervariable regions make contact with the antigen. In this process the CDR1 and CDR2 regions interact with the MHC-molecule on the antigen-presenting cell, while the CDR3 region bind to the peptide presented in the MHC molecule.

In addition to the 'first signal' of specific T-cell recognition of an antigen by the TCR, other cell surface structures play an important role in the activation process of the T-cell. These have been termed the 'second signals' (e.g. LFA1-ICAM1, CD2-LFA3, CD28-B7). Without these costimulatory signals, T-cells cannot respond to the presented antigen. Further to these signals, also cytokines have a major function in T-cell activation.

1.4. Antigen-presentation pathways

Recipient T-cells can recognise foreign donor MHC antigens by two different pathways, either directly as intact molecules or indirectly as peptides after antigen processing.

Direct antigen-presentation pathway

Recipient T-cells can directly engage and respond to intact allogeneic (donor) MHC molecules, either in combination with an allo-peptide or a self-peptide, on the surface of donor antigen-presenting cells (APCs). Direct T-cell activation is possible via two kinds of APCs: many tissues have their own APCs (e.g. Langerhans' cells in the skin, Kupffer cells in the liver), but also endothelial cells of vascularized grafts appear to have APC-function, especially if activated.[33] The normal T-cell repertoire contains a

very high precursor frequency of T-cells capable of responding to allogeneic HLA molecules.[34]

Indirect antigen-presentation pathway

T-cells can recognise nominal or recall antigens as processed peptides presented by self MHC molecules (MHC restriction). For recognition of allogeneic MHC via the indirect presentation pathway, donor MHC molecules are processed and presented by recipient MHC molecules on recipient APCs. Indirect presentation depends on the APCs in the recipient's draining lymph nodes and spleen. Dendritic cells and monocytes have the capability to stimulate indirect responses.[35,36] Also activated B-cells and T-cells can have APC function.[37,38]

Pathways of antigen processing

The MHC molecules present peptides to T-cells. Therefore, the antigens have to be segmented into peptides. Two types of responses can be mounted against pathogens that have invaded an organism.[39] Antigens derived from pathogens that have access to the cytosolic compartment of cells (e.g. viruses) are usually identified by the immune system via HLA class I molecules. Because these antigens are synthesized within the APC, it is also called the endogenous pathway. This pathway includes cytosolic proteins as well as membrane or secreted proteins. Whereas antigens that reside extracellularly (e.g. bacteria) are presented by MHC class II molecules. These antigens are internalized by the APC from the extracellular medium: the exogenous pathway.

MHC class I molecules bind peptides of 8 to 11 amino-acids long within the endoplasmic reticulum (ER). The peptides are generated by the proteasome and are translocated from the cytosol into the ER by TAP (transporters associated with antigen processing) molecules that are located in the ER-membrane. The peptide-MHC class I-complex travels through the Golgi-complex directly to the cell surface for presentation to $CD8^+$ T-cells.[40]

MHC class II molecules acquire peptides derived from antigens that are internalized in the endocytic pathway.[41] MHC class II heterodimers assemble in the ER with the invariant chain (Ii). At the trans-Golgi network the complex is targeted to the MHC class II compartments (MIIC) in the endocytic pathway. There the invariant chain is proteolytically removed and the antigenic peptides can bind to the class II molecules. Peptide loaded MHC class II complexes are then transported to the plasma membrane for presentation to $CD4^+$ T-cells.

In vitro data showed that also HLA class II molecules on APCs can present peptides derived from HLA class I[42,43] and class II[44,45] alloantigens.

Alloantigen recognition pathways after human transplantation

Most studies on the role of the direct and indirect presentation pathway in allograft

rejection have been performed in rodents. Only a few groups have investigated the presentation routes in human transplant recipients.
Shearers' group demonstrated in renal transplant recipients with stable graft function, that the direct pathway was not different from that of healthy volunteers. Those stable transplant patients showed, however, no response to alloantigens presented via the indirect pathway. The presence of reactivity via the indirect pathway was found to correlate with acute or chronic rejection in non-stable patients.[46,47] In addition, it has been suggested that the indirect pathway is more sensitive to immunosuppression than the direct route of alloantigen presentation.[47,48]
The group of Suciu-Foca studied the indirect immune response of kidney, heart and liver transplant recipients. They suggested that the indirect response to peptides of donor HLA antigens occur only to a limited number of HLA peptides and this is correlated with acute and chronic rejection.[49-54]

1.5. Cytokines after clinical heart transplantation

$CD4^+$ T-lymphocytes are the regulatory cells of the immune response. Much of their function is mediated through cytokines, whose expression and secretion are induced as a result of the engagement of the T-cell with an APC.

Type of cytokine producing T-helper cells
Mosmann[55] showed that murine $CD4^+$ T-cell clones can be separated based on the cytokines they produce in response to antigen. Early in the primary CD4 T-cell response a random assortment of cytokines (T-helper (Th) 0 cytokines) are produced. After prolonged stimulation, the $CD4^+$ T-cell population can be divided in a Th1 or Th2 population. Also $CD8^+$ T-cell population can be divided into Th1 and Th2 producing cells.[56,57] The Th1-like cells produce interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-β, while the Th2 pattern consists of IL-4, IL-5, IL-6 and IL-10 production. Th1 cells induce enhanced activity in macrophages, activation of cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells (cellular immunity), whereas Th2 cells make products that act on the B-cell which than differentiate it into an antibody-producing cell (humoral immunity). Both Th cell subsets tend to counteract and cross-regulate each other's activity. Therefore a popular hypothesis states that preferential induction of allograft-specific Th2 cytokines would inhibit Th1-regulated rejection responses.
In vivo T-cells are exposed to many different influences as they undergo primary activation, clonal expansion, effector cell differentiation, and reactivation by antigen. The cytokine expression pattern can be influenced by the outcome of interaction with APCs during priming, which can be affected by TCR affinity and avidity, peptide/MHC

density, costimulatory signals, the duration and frequency of interaction, the activities of bystander cells, cytokines, endocrine hormones, and other soluble mediators.[58] Therefore, the Th1/Th2 paradigm in man is not so strong, and it is not surprising that T-cells isolated from different tissue sites can display a diversity in cytokine profiles.

Monitoring of cytokines in peripheral blood after clinical heart transplantation

Jordan et al.[59] showed no correlation of IL-2 and TNF-α levels in serum of peripheral blood with the histological grade of allograft rejection. In agreement, Fyfe et al.[60] also demonstrated no relation with the level of IL-2, IL-4, IL-6 and TNF-α and the presence or absence of acute rejection both in serum from peripheral blood and from coronary sinus blood.

Despite these disappointing results, in 1996 another group tested levels of IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α in serum from peripheral blood from human cardiac allograft recipients.[61] These serum cytokine levels sometimes showed peaks that appeared to be correlated with acute rejection or occasionally to infection, but these relationships were not consistent. In contrast, in the same year, Chang[62] demonstrated again that monitoring cytokine levels of IL-1, IL-2 and TNF-α in serum is not useful in predicting cardiac transplant rejection.

Lagoo et al.[63] showed after rejection treatment a decrease in IL-1β, IL-6, and TNF-α expression in resting peripheral blood mononuclear cells (PBMC), and a decrease in IL-4 and IL-10 gene expression in phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) stimulated PBMC. They suggested that this decline was due to changes in the proportion of lymphocytes and monocytes in peripheral blood in concert with a steroid-induced downregulation by cytokine gene transcription.

Only one group reported a clear positive relation between cytokine levels in peripheral blood and acute rejection. Kimball et al.[64] suggested that high IL-6 and IL-8 levels, and not IL-1, IL-2 and TNF-α levels, are effective markers of acute rejection following human heart transplantation.

Intragraft monitoring of cytokines after clinical heart transplantation

Intragraft monitoring of cytokines gives better insight in the rejection process.

After clinical heart transplantation, Cunningham[65] demonstrated in endomyocardial biopsies (EMBs) that only the presence of IL-2 mRNA expression related to acute rejection, while IL-1β, IL-4, TNF-α, and TNF-β did not. Baan et al.[66] had also clearly demonstrated the importance of upregulation of the IL-2 gene in acute graft rejection, but also reported that IL-4 and IL-6 could play a role. Contrary to Cunningham et al. and Baan et al., Zhao et al.[67,68] found however no relation between IL-2, IL-4, IL-5, IL-1, TNF-α, and IFN-γ gene expression and acute rejection, but noted an increased IL-6 and TGF-β gene expression with rejection.

Both Baan et al.[66] and Azzawi et al.[69] found no relation with IL-10 and acute rejection. However, Grant et al.[70] suggested that IL-10 and possibly IL-4 may suppress graft

rejection, whereas IL-2 may promote rejection.
The cytokine protein can be detected with the use of monoclonal antibodies and immunohistochemical techniques.[71] In this way, the presence of IL-2 and IFN-γ located in lymphocytes of EMBs from cardiac allograft recipients was shown to be indicative of acute rejection, whereas IL-6 was rarely detected.[72]

1.6. Cytotoxic T-lymphocytes after clinical transplantation

The cytokines produced by T-helper cells play an important regulatory role in determining the activation state of the cytotoxic T-lymphocytes (CTLs).
To destroy allogeneic target cells, the target cell is recognized by T-cells using their specific TCR and other receptor-ligand interactions (e.g. CD8-HLA class I, CD4-HLA class II, CD2-LFA3, LFA1-ICAM1). In cytotoxic reactions the attacking cells direct their granules towards the target cell. The granules of the CTLs contain perforins and granzymes, which can punch holes in the outer membrane of the target cell. CTLs can also signal to the target cell to start a programme of self-destruction resulting in fragmentation of the DNA in a process called apoptosis.

During stable engraftment, Mathew et al.[73] and Van Twuyver et al.[74] demonstrated donor-specific CTL non-responsiveness in the peripheral circulation of long term liver allograft recipients, while this is not common after kidney transplantation.[74]
Hu et al.[75] showed 4 to 6 months after heart transplantation a decrease in donor-specific circulating CTLs. Steinmann et al.[76] found the same reduction within the first 3 months after kidney transplantation. The reduction of CTLs was seen in patients with acute rejection episodes as well as in patients without rejection. Vaessen et al.[77] could not detect a correlation with CTLs in peripheral blood and rejection, while the number of committed donor-specific CTLs propagated from EMB-cultures correlated well with acute rejection.
From literature, it is clear that CTLs can destroy allografts,[78] however CTLs can also be obtained from stable, well functioning allografts.[79,80] Therefore, the difference in quality between CTLs derived from non-rejecting and rejecting grafts may play a major role in the rejection process.
CD8 and CD4 molecules on the T-lymphocyte are accessory molecules that stabilize TCR-antigen interaction by binding to the non-polymorphic region of HLA class I and class II, respectively.[81,82] CTLs that are dependent on CD8 or CD4 molecules are known as low avidity cells. In the presence of CD8 or CD4 monoclonal antibodies these CTLs are not able to lyse their target cells, whereas CTLs with high avidity remain effective in this area.
The number of high avidity CTLs directed to donor HLA class I antigens is higher in

patients with acute rejection than those who had never experienced rejection,[83] this number even increases during a rejection period.[84] The importance of HLA class II directed CTLs has also been demonstrated in relation to human cardiac allograft rejection.[85] In addition to high avidity CD8$^+$ CTLs, CD4$^+$ CTLs can mediate heart allograft rejection when they have high avidity for HLA class II donor antigens.[86]

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Chapter 2

Aim of the study

The main aim of the studies described in this thesis is the monitoring of T-cell reactivity in relation to acute rejection and graft vascular disease (GVD) after heart transplantation. These rejection processes are investigated both in peripheral blood leukocytes and in graft-infiltrating lymphocytes.

2.1. Peripheral blood monitoring after clinical transplantation

In addition to testing of the direct and indirect presentation pathways *(chapter 1.4)* and the inventory of cytokine production *(chapter 1.5)*, various other *in vitro* techniques with peripheral blood have been investigated for a possible role in monitoring graft rejection.

Variable results were obtained with the mixed lymphocyte culture (MLC). Langhoff et al.[1] suggested a primary role for MLC matching in the outcome of kidney graft survival. This was supported by the observation that the MLC correlated well with DR-matching. Additionally, evidence was presented for a role of the MLC to detect donor-specific hyporesponsiveness. Ghobrial et al.[2] showed that in some renal allograft recipients, a state of donor-specific hyporesponsiveness develops, and this state may be associated with a better graft outcome (less acute rejection episodes) at one year. The same was demonstrated for liver, lung and kidney transplant patients.[3] These hyporeactive patients also experienced a lower incidence of chronic rejection.[4,5] In contrast to these observations, no correlation between the MLC and graft outcome in patients receiving a renal allograft was found by Cullen et al.[6] and Jeffery et al.[7]

Limiting dilution assays have also been used in peripheral blood monitoring to correlate the frequency of helper T-lymphocyte precursors (HTLp) and cytotoxic T-lymphocyte precursors (CTLp) with clinical relevant parameters. A significantly reduced proportion of HTLp in the peripheral blood mononuclear cell population from immunosuppressed allograft recipients was demonstrated by Cattell et al.[8] However, it was still possible to measure the frequency of donor-reactive helper cells in the blood cells of renal and cardiac transplant patients.

The frequency of donor-specific HTLp increased during episodes of acute rejection after heart transplantation.[9] Zanker et al.[10] showed that after kidney transplantation functional deletion of circulating donor-reactive T-cells can occur at the level of HTLp, but also at the level of CTLp, or both. Reader et al.[11] found an increase of CTLp frequencies during acute cardiac allograft rejection, which could not be confirmed by others.[12]

2.2. Monitoring of graft-infiltrating lymphocytes in relation to acute rejection and graft vascular disease after clinical heart transplantation

Although macrophages, B-lymphocytes, neutrophils, eosinophils and natural killer cells have been identified in rejecting grafts,[13,14] it is generally accepted that T-lymphocytes are the primary mediators of graft rejection.[15] T-lymphocytes that infiltrate the allograft, are primed to donor antigens and undergo expansion and functional differentiation. Studying their characteristics may be helpful in understanding the development of acute rejection and GVD.

Immunohistochemical studies of lymphoid infiltrates, have shown that $CD3^+$ lymphocytes are the predominant cell type. Both $CD4^+$ and $CD8^+$ cells are generally present. Infiltrating T-cells express IL-2 receptors and HLA-DR antigens.[16]

An elegant approach in studying the functional characteristics of the graft-infiltrating lymphocytes (GIL) is to isolate these cells from the graft. The *in vitro* propagation of lymphocytes from human transplant biopsies is based upon the concept that the allograft is infiltrated by activated T-lymphocytes that proliferate in the presence of exogenous IL-2.[17] Activation of T-cells occurs as a result of recipient allorecognition of donor antigens which lead to the expression of IL-2 receptors on the activated T-cells. Subsequently, IL-2 is required for clonal expansion.

From all heart recipients transplanted in the University Hospital Rotterdam-Dijkzigt, peripheral blood cells and endomyocardial biopsies (EMBs) were collected, while spleen cells were extracted from the original heart donor. The EMBs were cultured in IL-2 containing medium. When GIL-cultures were obtained, their phenotype was determined and a cell mediated lympholysis assay was performed to measure their cytotoxic capacity against donor antigens. After this analysis the remaining cells were stored at -140°C.

Successful cell growth from EMBs was cross-checked with the histologic rejection grade and time-lapse after transplantation. Cell growth was observed to be more frequent during an acute rejection episode than in rejection-free periods.[18,19] In addition, *in vivo* activated, committed donor-specific CTLs were propagated from expanded GIL-cultures and their presence was linked to acute rejection, while in peripheral blood no committed CTLs were found.[20] Based on these results, the importance of investigating GIL-cultures is demonstrated. During further investigations, the phenotype of the cultures appeared to correlate with acute rejection: more $CD4^+$ T-lymphocytes were cultured from rejection-EMBs than from those which remained free from rejection.[19]

During the first 3 months after transplantation, an increased number of cultures from patients who experienced one or more rejection episodes had cytotoxic reactivity recorded against both donor HLA class I and class II antigens versus patients who

did not have signs of rejection.[18,19] The frequency of committed CTLs increased significantly towards rejection and recovered to a base level after rejection treatment. In contrast, no significant difference was found in the number of precursor CTLs.[21]
The avidity of the CTLs for antigen proved to be an important characteristic in relation to acute rejection. The cytotoxic capacity of CTLs with low avidity for alloantigens can be inhibited with CD8 or CD4 monoclonal antibodies, while high avidity CTLs are not affected. In patients with rejection, the number of high avidity CTLs (CD8 and CD4 resistant) was higher than in patients who had never experienced rejection.[22,23] Before rejection episodes, only a small fraction of the CTLs had high avidity for donor antigens, while this fraction increased gradually and became dominant during rejection. After successful rejection treatment a decrease to base levels was observed.[21]
The characteristics of the GIL-cultures were also investigated in relation to the onset of GVD. The incidence of acute rejection, the period between transplantation and the first rejection episode as well as the successful cell growth from EMBs were not different between patients with and without signs of GVD.[24] During the first year after transplantation patients with signs of GVD had a higher percentage of $CD8^+$ T-cells and a lower percentage of $CD4^+$ T-cells in their GIL-cultures.[25] Although, the cytotoxic reactivity of the cultures against donor HLA class I or class II antigens was comparable between the patient groups.

2.3. Objectives of the study

The theme of this thesis is intragraft (*chapter 3 and 4*) and peripheral blood (*chapter 5*) immunological monitoring in relation to both acute rejection (*chapter 3 and 5*) and GVD (*chapter 4 and 5*) in an endeavour to understand and to interpret the immunological mechanisms and processes during both acute rejection and GVD. In addition, to establish a pattern and identify key characteristics in acute rejection and the development of GVD.

The results described in *chapter 2.2* suggest that allograft-infiltrating lymphocytes play a key role in acute rejection as well as in the development of GVD. Therefore both rejection processes were studied in graft-infiltrating lymphocytes (*chapter 3 and 4*).
Chapter 3 describes some characteristics of lymphocytes that infiltrate the rejecting cardiac allograft. The cell growth and phenotype of the GIL-cultures propagated from EMBs taken during an acute rejection episode and during a rejection-free period are investigated (*chapter 3.1*), which extends previous observations by Ouwehand et

al.[18,19] Thereafter, the local production of cytokines by T-helper 1 and T-helper 2 cells within the lymphocyte cultures derived from rejection EMBs and non-rejection EMBs is described (*chapter 3.4*). Special attention is given to the concordance (*chapter 3.2*) and kinetics (*chapter 3.3*) of cytokine gene expression and protein production.
Chapter 4.1 demonstrates patterns of cell growth and phenotypic characteristics of GIL-cultures derived from patients with and without GVD in different periods after heart transplantation, which extends previous studies of Groeneveld et al.[24] and Jutte et al.[25]
Chapter 4.2 studies whether there is a relationship between levels of T-helper 1 and T-helper 2 cytokines produced by the infiltrating lymphocytes and the process of the onset of GVD. In *chapter 4.3* the cytotoxic capacity of the GIL-cultures to donor HLA class I and class II antigens and their relation to GVD is also described. The second part of this chapter discusses the relevance of $CD8^+$ and $CD4^+$ CTLs during the development of GVD using the frequency and avidity of committed CTL for donor HLA class I and class II antigens.

Because the EMB-procedure is an invasive method and propagating GIL-cultures is time consuming, also relations with peripheral blood monitoring and acute rejection and the development of GVD are investigated (*chapter 5*).
Only two groups (*chapter 1.4*) have studied alloantigen recognition pathways after human transplantation. The importance of the direct and indirect pathway remains unclear. Accordingly, *chapter 5.1* reports the response of peripheral blood cells to antigens presented via the direct and the indirect pathway. Both presentation pathways are not only related to acute rejection and GVD, but also periods of immunological quiescence are investigated.
An increased frequency of circulating HTL[9] and CTL[11] was related to acute rejection after heart transplantation. Their role in the development of GVD is unknown. Therefore in *chapter 5.2* donor-specific HTL and CTL frequencies are measured in peripheral blood and related to GVD.

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Chapter 3

Intragraft monitoring of acute rejection

Chapter 3.1

Phenotypic analysis of lymphocytes infiltrating human cardiac allografts during acute rejection

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Abstract

Acute rejection (AR) is mediated, at least in part, by cellular processes. Therefore, we cultured graft-infiltrating lymphocytes (GIL) from endomyocardial biopsies (EMB) taken during the first year after transplantation, determined their phenotypic composition, and correlated it to AR. We observed more often GIL growth from EMB with AR than from non-rejection EMB (P=0.02). $CD4^+$ cells were always more numerous than $CD8^+$ cells, and no difference in phenotypic composition was found between AR and non-rejection EMB.

In conclusion, AR is correlated with cell growth of EMB and not with the phenotypic composition.

Introduction

In several studies, we and others have shown that alloactivated graft-infiltrating lymphocytes (GIL) can be expanded *in vitro* from endomyocardial biopsies (EMB) in IL-2-containing medium.[1,2] This culture system is thought to promote growth from *in vivo* activated GIL only.[2] We also have suggested that the outgrowth of GIL from EMB and their phenotypic composition corresponded with histological findings of acute rejection (AR).[1] In the present study, we applied the same method of culturing GIL, and studied per patient the proportion of EMB from which GIL could be obtained and determined their phenotype in the first 3 months after heart transplantation (HTx), in an attempt to show whether our previous findings in groups of patients could be applied to individual cardiac transplant recipients. We compared these parameters for episodes with and without AR.

Patients and methods

We studied 60 cardiac allograft recipients, who were transplanted between March 1991 and September 1993. EMB were taken at regular intervals after HTx and examined histologically. According to the criteria of the International Society of Heart and Lung Transplantation,[3] AR was defined as cell infiltrate with myocyte necrosis and anti-rejection therapy was prescribed.

Culture method and phenotypic analysis

We received EMB from 49 out of 60 patients taken in the first 3 postoperative months to obtain GIL. GIL were cultured from EMB in IL-2 containing medium as described before.[1] GIL were analyzed by two-colour flow cytometry on a FACScan for the expression of cell surface markers. Initial screening was performed with the combinations WT31 FITC-CD3 PE, CD4 FITC-CD8 PE, and CD16 FITC-CD56 PE. When $CD3^{+}WT31^{-}$ cells were found, the GIL cultures were stained for T-cells bearing T-cell receptor (TCR)-γδ. WT31 (TCR-αß) and TCR-γδ were obtained from Becton Dickinson (San Jose, CA, USA) and the other monoclonal antibodies from Immunotech (Marseille, France).

Statistics

We determined from all individual patients the fraction of EMB in the first 3 postoperative months showing signs of AR, the GIL growth from EMB, and their phenotypic composition. The Mann-Whitney U test was used to evaluate the differences between the patient groups.

Results

Most acute rejections occur in the first 3 months after HTx.[4] Therefore, we analyzed the relationship between AR and outgrowth of GIL from EMB and the percentage of phenotypic composition in this period.

More outgrowth of GIL from EMB was seen when AR was diagnosed (Table 1).

The same quantities of TCR-αß, TCR-γδ, CD16, CD3, CD4 and CD8 were found in the GIL derived from rejection and non-rejection EMB (Table 1). Significantly more

CD4^{+} than CD8^{+} T-lymphocytes were obtained from EMB with and without AR (P=0.001).

Table 1 Graft-infiltrating lymphocyte (GIL) outgrowth from endomyocardial biopsies (EMB) and their phenotypic composition of non-rejection and rejection EMB taken from 49 patients in the first 3 months after heart transplantation (HTx).

	non-rejection (mean %)[a]	rejection (mean %)[a]	P-value
cell growth	42	61	0.02
TCR-αß	90	94	0.14
TCR-γδ	2	1	0.74
CD3	92	94	0.37
CD16	8	4	0.14
CD4	57[b]	59[c]	0.57
CD8	32[b]	35[c]	0.64

[a]Mean value of all individual patients
[b,c]Mean percentage CD4 versus CD8 in cultures derived from non-rejection (P<0.001)[a] and rejection (P=0.001)[b] EMB.

Discussion

The positive correlation between cell growth from EMB and AR is in agreement with our previous study which described the results per EMB taken from cardiac allograft recipients,[1] and can be explained by the higher frequencies of cells infiltrating the rejecting allograft compared to non-rejecting grafts. In the previous study[1] and this report, we found CD4 to be the predominant phenotype in most cultures. In contrast, in individual patients we could not confirm that cultures derived from EMB with signs of AR were associated with a higher proportion of CD4-dominated cultures.[1] However, we analyzed the phenotypic composition per patient in a fixed period after transplantation (0-3 months) and not individual GIL cultures. Another difference between the earlier report and the present results is that we investigated rejection and non-rejection EMB per individual patient and not EMB derived from groups of patients with and without rejection episodes.

From the described study, we conclude that AR is positively related to GIL growth from EMB, and there is no relation to the phenotypic composition of the GIL and AR.

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Chapter 3.2

Concordance of mRNA expression and protein production of IL-2 and IL-4 by human heart graft-infiltrating lymphocytes

Nicole M. van Besouw, Cornelis R. Daane, Carla C. Baan, Wendy M. Mol, Lenard M.B. Vaessen, Hubert G.M. Niesters, Nicolet H.P.M. Jutte, and Willem Weimar.

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Introduction

Cytokines play a central role in the induction of alloreactive effector cells. Several groups have studied the production of cytokines, their receptors, and the corresponding mRNA in peripheral blood or directly in the transplanted organ (reviewed by Dallman).[1] Analysis of mRNA gene expression and the production of cytokines from *in vitro*-propagated graft-infiltrating lymphocytes (GIL) may give information how cytokine secretion within the transplanted heart is regulated.

Materials and methods

GIL cultures propagated from human endomyocardial biopsies were established in medium containing interleukin-2 (IL-2).[2] Before testing, the cells were washed and $5x10^4$ cells/well were incubated in IL-2-free medium during 24 hours. Thereafter, $5x10^4$ irradiated (60 Gy) and washed Epstein-Barr virus (EBV)-transformed B-cells (B-LCL) of donor origin or a third-party were added.
After 4 hours of coculturing, cell pellets were snap-frozen for mRNA analysis by reverse transcriptase PCR.[3] Sequence-specific primers were used for amplification of the human cytokine genes (Table 1). ß-Actin served as positive control for successful RNA extraction.
In parallel, B-LCL were cocultured for 20 hours and the concentration of IL-2, IFN-γ, and IL-4 in the supernatants were determined by ELISA (IL-2: detection range 15 to 1000 pg/ml, IFN-γ: range 80 to 5000 pg/ml and IL-4: range 10 to 450 pg/ml).

Table 1 Oligonucleotides used to amplify target cDNA. Product sizes predicted, and the sequences of the internal oligonuleotides used for Southern blot analysis of the products.

Target	Sequence	Product size (bp)
IL-2 sense	5' ATG TAC AGG ATG CAA CTC CTG TCT T 3'	458
IL-2 anti-sense	5' GTC AGT GTT GAG ATG ATG CTT TGA C 3'	
IL-2 probe	5' TTC TTC TAG ACA CTG AAG ATG TTT CAG TTC 3'	
IFN-γ sense	5' TCT GCA TCG TTT TGG GTT CT 3'	301
IFN-γ anti-sense	5' CAG CTT TTC GAA GTC ATC TC 3'	
IFN-γ probe	5' TCT GGT CAT CTT TAA AGT TTT TAA AAA GTT 3'	
IL-4 sense	5' ATG GGT CTC ACC TCC CAA CTG 3'	462
IL-4 anti-sense	5' TCA GCT CGA ACA CTT TGA ATA TTT CTC TCT CAT 3'	
IL-4 probe	5' GTC CTT CTC ATG GTG GCT GTA GAA CTG CCG 3'	
β-actin sense	5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG 3'	838
β-actin anti-sense	5' CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC 3'	
β-actin probe	5' GGC TGG GGT GTT GAA GGT CTC AAA CAT GAT 3'	

Results and discussion

Unstimulated GIL cultures and stimulator B-LCL alone neither expressed IL-2 or IL-4 mRNA nor produced these cytokines. In contrast, IFN-γ mRNA was present in these controls, although no protein was detected.
Table 2 summarizes the results obtained from stimulated GIL cultures. When IL-2 and IL-4 mRNA were expressed in cells after 4 hours of stimulation, we always found the corresponding cytokine protein in supernatants after 20 hours of stimulation. In case of IFN-γ, the mRNA was always present; however, subsequent protein production was only found after donor stimulation. Quantitative PCR may demonstrate that the IFN-γ mRNA expression after donor stimulation is higher than after third-party stimulation or unstimulated control cells alone. A clear donor-specific reaction was only seen for IL-2 mRNA expression and the protein production of IL-2 and IFN-γ. In a larger group of GIL cultures, we described the donor specificity of cytokine production and the predominance of Th1 cytokines after donor stimulation.[4]
In conclusion, the present study showed a clear concordance between mRNA transcription of IL-2 and IL-4 after 4 hours of stimulation and protein production 20 hours after stimulation.

Table 2 mRNA expression and production of cytokines by seven graft-infiltrating lymphocyte (GIL) cultures, obtained from endomyocardial biopsies of six cardiac transplant recipients, after stimulation with donor B-cell lines (B-LCL) or with third-party B-LCL.

	IL-2		IFN-γ		IL-4	
	donor	3rd party	donor	3rd party	donor	3rd party
mRNA@ expression	7/7	3/7*	7/7	7/7	2/7#	1/7
protein@ production	7/7	3/7*	7/7	3/7*	2/7#	1/7

@The GIL cultures were scored for the presence of mRNA expression or protein production.
*P=0.08; donor response compared with third-party response (chi-square test with Yates correction).
#P=0.03; Th1 (IL-2 and IFN-γ) response compared with Th2 (IL-4) response for both mRNA expression and protein production (chi-square test with Yates correction).

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Chapter 3.3

Kinetics of IL-2 and IL-4 mRNA and protein production by graft-infiltrating lymphocytes responsible for rejection after clinical heart transplantation

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Abstract

During cardiac rejection we studied the kinetics of IL-2 and IL-4 mRNA and subsequent protein production by *in vivo* primed graft-infiltrating lymphocytes (GIL), using semi-quantitative RT-PCR and ELISA. Following *in vitro* stimulation with either donor or third-party antigens, mRNA expression of IL-2 and IL-4 were already detectable 1-2 h after stimulation, while their protein production could be measured from 4 h onwards at least until 48 h. At both the mRNA and protein level, we measured a donor-specific signal for IL-2 and for IL-4 production (P=0.02), while the relative donor-specific IL-2 mRNA level was significantly higher than the relative IL-4 mRNA level (P=0.002). These observations suggest that after *in vitro* challenge with donor antigens, GIL obtained from rejecting cardiac allografts predominantly produce IL-2 mRNA and protein.

Introduction

After clinical heart transplantation, acute rejection usually occurs within the first three months and is characterized by high levels of inflammation in the graft.[1-3] The immune mechanisms responsible for cellular allograft rejection are dependent on the activities of primed allospecific T-lymphocytes. Interaction of these immunocompetent $CD4^+$ T helper cells (Th) and $CD8^+$ cytotoxic T-lymphocytes (Tc) with donor antigens results in activation of the transcription of cytokine messenger RNAs (mRNA) followed by the subsequent cytokine protein release. These soluble mediators regulate the immune response against the transplanted graft.
Human T-cells, although not so tightly restricted as described for mouse T-cells, can be divided into functionally different subsets on basis of their cytokine production pattern. Th0 cells produce cytokines such as IL-2 and IL-4, while Th1/Tc1 cells secrete IL-2, IFN-γ, TNF-ß and Th2/Tc2 cells produce IL-4, IL-5, IL-6, IL-10.[4,5]
Allograft rejection is associated with an upregulation of several cytokines within the graft implying that Th0 cells or a mixture of both Th1/Tc1 and Th2/Tc2 cells have infiltrated the allograft.[6,7] The production of cytokines within the graft can be analysed directly in biopsies on the transcriptional level and on the protein level using techniques such as RT-PCR and immunohistochemistry.
It was shown that, in man, cardiac allograft rejection is associated with intragraft production of IL-2 and IFN-γ at both the transcriptional and protein level.[8-10] In contrast, for the Th2 cytokines IL-4 and IL-10 the data are less clear. Also at the transcriptional level, we and others found that both during acute cardiac allograft rejection and during immunological quiescence IL-4 and IL-10 mRNA expression can be found within the graft.[8,9] This suggests that while these cytokines may be present during allograft rejection, they actually may be involved in the down-regulation of the allogeneic immune response. Indeed, a role for IL-4 in the process of spontaneously resolving rejection and even in tolerance has already been reported after clinical liver and renal transplantation.[11,12]
Although it is thus clear that both IL-2 and IL-4 are important determinants of outcome of the immune process in human transplants, the kinetics of Th1 and Th2 cytokine production by graft-infiltrating lymphocytes (GIL) at the mRNA and at the protein level in the graft are unknown. The capacity of *in vivo* primed GIL to produce cytokines can be determined after *in vitro* challenge with donor antigens.[13] In order to study the regulation of cytokine production by GIL, it is essential to measure cytokine mRNA expression, since cytokine production is predominantly controlled at the transcriptional level.[14] An important advantage of our *in vitro* system is that mRNA expression by *in vivo* primed GIL can be compared directly with the cytokine protein production. Therefore, we have analysed the kinetic pattern of IL-2 and IL-4 mRNA synthesis by *in vivo* primed GIL, grown from rejection biopsies, after *in vitro*

stimulation with donor and third-party antigens using semi-quantitative RT-PCR analysis. These findings are compared directly with levels of these cytokines measured in cell culture supernatants by ELISA.

Materials and methods

Patients
Endomyocardial biopsies were obtained from three heart transplant recipients during their first acute clinical rejection episode (14-84 days post-transplant). Maintenance immunosuppressive therapy consisted of cyclosporin A and low-dose steroids. No additional therapy was given. Rejection was histologically diagnosed according to the criteria of the International Society for Heart and Lung Transplantation.[3] For the diagnosis of clinical significant rejection, the coexistence of myocyte damage and mononuclear infiltrates was required (grade 3A). In that case, anti-rejection therapy was instituted.

GIL culture method
GIL cultures were grown from endomyocardial biopsies in 96-wells U-bottom tissue-culture plates (Costar, Cambridge, MA, USA) as described before.[15] GIL were expanded in culture medium (RPMI-1640 Dutch Modification Gibco, Paisley, UK) supplemented with 10% pooled human heat-inactivated serum, 4 mM L-glutamine, 100 IU penicillin and 100 μg/ml streptomycin) in the presence of 30 U/ml exogenous IL-2 (lectin-free lymphocult T-LF, Biotest AG, Dreieich, Germany) and 10^5 irradiated (30 Gy autologous peripheral blood mononuclear cells. GIL cultures were grown at 37 °C in a humidified CO_2 incubator for two to three weeks. Half of the medium was refreshed every two to three days.

Phenotypic analysis of GIL cultures
Surface antigens were analysed by two-color flow cytometry after staining with monoclonal antibodies directed against CD3 (anti-leu 4) as pan mature T-cell marker, WT31 as a marker for the α/ß-chain and 11F2 as a marker for γ/δ-chain of the T-cell receptor. CD4 (anti-leu 3) and CD8 (anti-leu 2) were used as T-cell subset markers. CD16 (anti-leu 11) was used as a marker for natural killer cells. The antibodies were directly conjugated to fluorescein or phycoerythrin (Becton and Dickinson, Maintain View, CA, USA). GIL cultures were analysed using a flow cytometer (FACscan, Becton and Dickinson).

Allogeneic stimulator cells
B lymphoblastoid cell lines (B-LCL) originated from the infection of donor and third-party spleen cells with EBV obtained from the marmoset cell line B95-8.[16]

Study design
Before testing, GIL were washed and $5x10^4$ cells/well were incubated in IL-2 free culture medium for 24 h. Thereafter, $5x10^4$ irradiated (60 Gy) and washed B-LCL of donor or third-party control origin were added. Third-party control B-LCL shared human leucocyte antigens neither with the donor nor with the recipient. At each time-point, the GIL were stimulated in duplo. After stimulation, these cell pellets and supernatants were pooled. Supernatants (180 μl) and cell pellets (10^5 GIL + 10^5 irradiated stimulator cells) were harvested between 1 and 72 h of co-culture and stored at -80°C before analysis of cytokine production. Washed unstimulated GIL and irradiated stimulator B-LCL alone served as control.

Total RNA preparation
Total RNA was isolated from the stimulated GIL and their controls by a modification of the Chomczynski and Sacchi method as described before.[9,17,18] Briefly, cells were homogenized in 500 μl

4 M guanidinium-isothiocyanate in the presence of 20 µg poly A (Boehringer Mannheim, Germany). The solution was extracted once with phenol, phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1), respectively. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000*xg* at 4°C and washed once with 500 µl ice-cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpyrocarbonate-treated H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice.

cDNA synthesis

First strand cDNA synthesis was performed from 25 µl of the isolated RNA with 0.25 µg hexanucleotides (Promega Corporation, Madison, WI, USA) and transcribed with 500 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) at 42°C for 90 min in a total volume of 50 µl. The reaction mixture contained 10 µl 5x MMLV-RT buffer (250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 375 mM KCl), 2.5 µl dNTP (10 mM), 200 U of RNAsin (Promega) and 5 µl (0.1 M) DTT.

PCR amplification and Southern blot analysis

Sequence-specific primers were used for amplification of the human cytokine genes (Table 1). PCR primers detecting transcripts for the human house keeping gene keratin, which is expressed by haematopoietic cells,[19] were used as an internal control to monitor mRNA extraction and cDNA amplification (Table 1). For qualitative analysis, 5 µl cDNA sample was added to 95 µl PCR mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5' and 3' sequence-specific primers. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St. Louis, MO, USA) prior to PCR reaction in a DNA thermal cycler (Biomed-60, Germany) under the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min at 72°C. Positive control samples were produced by stimulating 10^6 human spleen cells with 1% phytohemagglutinin (PHA)-M (Difco, Detroit, MI, USA) for 4 h at 37°C. Messenger RNA from this positive control was extracted as described above. Negative controls consisted of omission of reverse transcriptase from the cDNA synthesis reaction for each EMB followed by amplification in PCR with the IL-2, IL-4 and keratin primers, and the use of and diethylpyrocarbonate-treated H_2O as no-template reaction. After amplification, 16 µl PCR product was electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, Aylesbury, UK) by electroblotting and hybridized with γ^{32}P-labelled specific probes. Hybridization was detected by autoradiography.

Semi-quantitative RT-PCR

For semi-quantitative analysis, cDNA samples were titrated (10-fold dilutions) and aliquots of each dilution were amplified using the conditions as described above. The amount of target cDNA present was expressed as end-point of the titration. Corrections were made for the integrity of the mRNA isolated and the efficiency of cDNA synthesis, which both may vary from isolation to isolation by standardization with keratin. It was expressed as arbitrary mRNA equivalents, defined as the highest dilution showing positive signal. To estimate the relative initial amount of functional mRNA in the GIL cultures, aliquots of titrated cDNA samples were amplified using specific primers for keratin to correct for the above mentioned variables. The results were expressed as a ratio calculated from the titre of IL-2 or IL-4 gene PCR product over the titre of amplified keratin product. To exclude inter- and intra-assay variability, amplification of titrated cDNA of PHA-M activated spleen cells were included in each assay. These control cells expressed keratin, IL-2 and IL-4.

Enzyme linked immunosorbent assay (ELISA)

The concentration of cytokines in the supernatants of the GIL cultures was measured with the following ELISA kits: IL-2 (Immunotech, Marseille, France) detection range 15-1000 pg/ml IL-2; IL-4

(Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) detection range 10-450 pg/ml IL-4.

Statistics
As data regarding relative levels of mRNA expression and secreted IL-2 and IL-4 in supernatants were nonparametric, pairwise comparisons between groups were carried out using the Wilcoxon signed rank test. All P-values ≤0.05 were considered to be statistically significant.

Table 1 Oligonucleotides used to amplify target cDNA. Product sizes predicted, and the sequences of the internal oligonuleotides used for Southern blot analysis of the products.

Target	Sequence	Product size (bp)
IL-2 sense	5' ATG TAC AGG ATG CAA CTC CTG TCT T 3'	458
IL-2 anti-sense	5' GTC AGT GTT GAG ATG ATG CTT TGA C 3'	
IL-2 probe	5' TTC TTC TAG ACA CTG AAG ATG TTT CAG TTC 3'	
IL-4 sense	5' ATG GGT CTC ACC TCC CAA CTG 3'	462
IL-4 anti-sense	5' TCA GCT CGA ACA CTT TGA ATA TTT CTC TCT CAT 3'	
IL-4 probe	5' GTC CTT CTC ATG GTG GCT GTA GAA CTG CCG 3'	
Keratin sense	5' TGA AGA TCC GTG ACT GGT AC 3'	218
Keratin anti-sense	5' ATG TCG GCT TCC ACA CTC AT 3'	
Keratin probe	5' TCT CCT TCT GCA GAT TGA CAA TGC CCG TCT 3'	

Results

Phenotypic analysis of GIL cultures
Phenotypic characterization of the three GIL cultures showed that the majority of the cells of the GIL cultures were $CD3^+$ (95%, 100%, 100%) and expressed the αß T-cell receptor. In these cultures 29%, 52%, 61% of the cells expressed CD4 and 39%, 40%, 70% CD8. In culture I CD16 (5%) positive cells were present.

mRNA expression of the housekeeping gene keratin
This transcript was chosen as a marker for the total number of cells and for the total quantity of mRNA extracted. Keratin mRNA expression is found to be in the range of mRNA expression of the cytokines tested, which is an advantage above the frequently used house keeping genes ß-actin and glyceraldehyde 3-phosphate dehydrogenase.[18,20] Especially in muscle tissue glyceraldehyde 3-phosphate dehydrogenase levels are abundant and therefore not suitable for analysis in heart samples.[21]
All samples obtained from one culture were isolated at the same time. After activation with either donor or third-party antigens, the keratin mRNA levels gradually

increased (Figure 1). Semi-quantitative analysis showed a maximum increase of the keratin mRNA levels of one-dilution step primary between 1 h and 72 h after stimulation. However, there was no statistically significant difference in the amount of mRNA for keratin after donor or third-party stimulation (P=0.50).

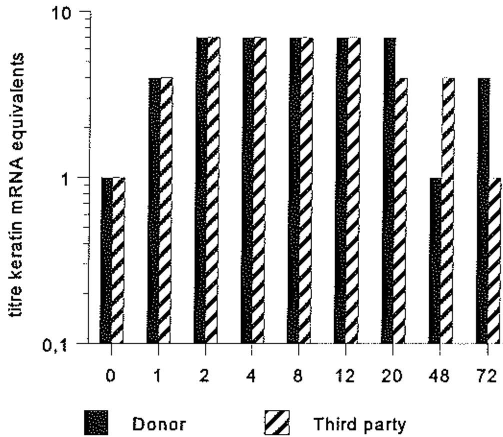

Figure 1 Time-course (in hours) of keratin mRNA expression in GIL cultures (n=3) after stimulation with either donor or third-party antigens. To estimate the relative initial amount of target cDNA, aliquots of 10-fold diluted cDNA samples were amplified using specific primers for keratin. After amplification the amount of target cDNA was determined by the highest dilution showing a positive signal after hybridization. Each bar represents the relative mean value (± SD) for three GIL cultures.

Relative IL-2 and IL-4 mRNA levels by GIL cultures

In the present study, IL-2 and IL-4 mRNA expression was analysed only in GIL cultures and irradiated stimulator B-LCL with keratin mRNA expression.

Figure 2 depicts the time-course of keratin, IL-2 and IL-4 mRNA expression in culture III after stimulation with donor and third-party cells.

Table 2 shows the kinetics of IL-2 and IL-4 mRNA expression of each individual GIL culture after stimulation with donor and third-party antigens, respectively.

Unstimulated GIL cultures (0 h), and irradiated stimulator B-LCL alone neither expressed mRNA nor secreted protein of IL-2 and IL-4 at detectable levels (Tables 2 and 3).

On stimulation by cells of donor or third-party origin an upregulation of cytokine mRNA production was observed. In general, IL-2 and IL-4 mRNA was apparent as early as 1 h after stimulation and reached maximal levels within 2-48 h after which it gradually decreased to prechallange base-line levels (20-72 h). This pattern was

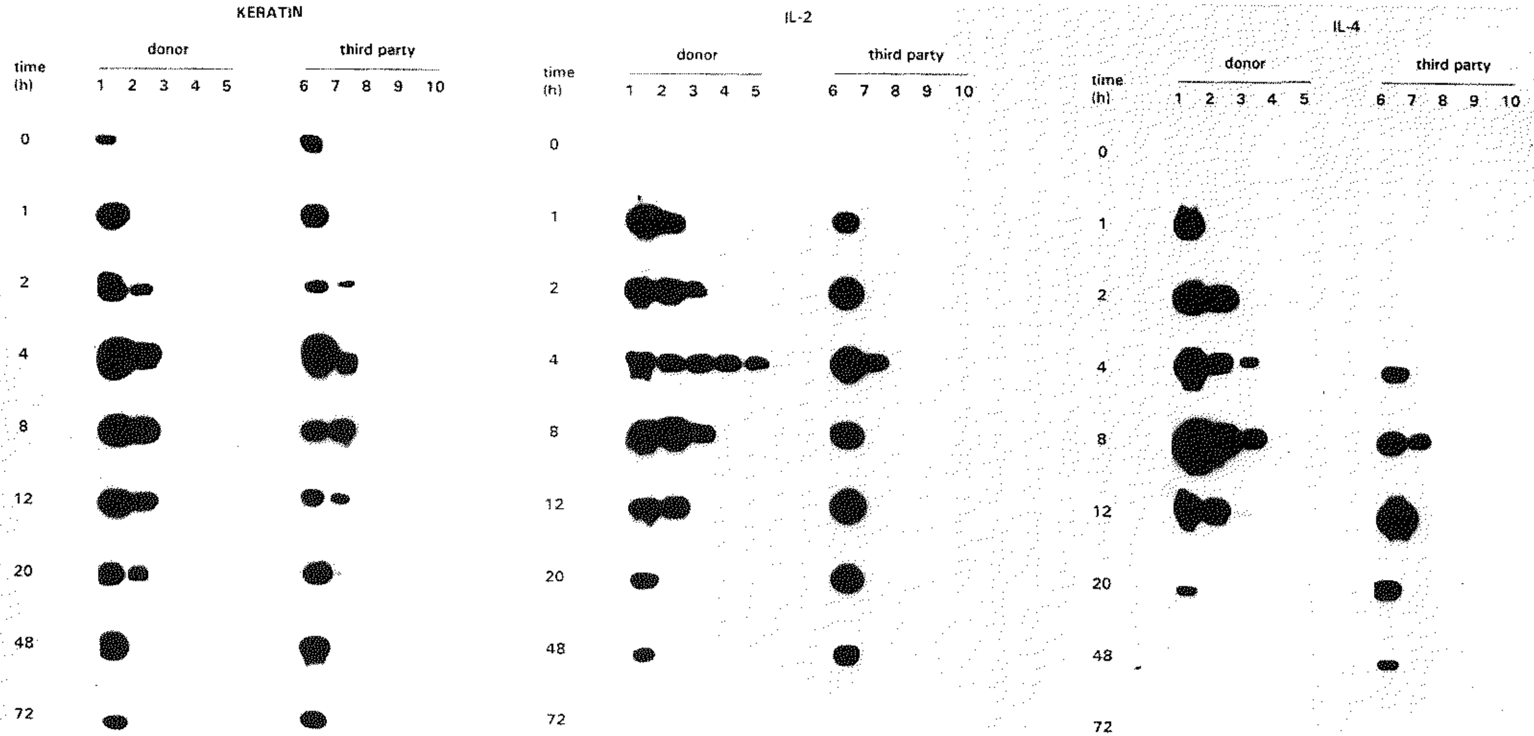

Figure 2 Semi-quantitative RT-PCR analysis of keratin (A), IL-2 (B) and IL-4 (C) mRNA expression by Southern blotting and hybridization. Time course (in hours) of culture III after stimulation with donor cells (lane 1-5) and third party cells (lane 6-10). Serial dilution of 10-fold diluted cDNA samples. Lane 1, undiluted cDNA after stimulation with donor cells; lane 2, 10-fold diluted; lane 3, 100-fold diluted; lane 4, 1000-fold diluted; 10000, 10000-fold diluted cDNA. Lane 6, undiluted cDNA after stimulation with third party cells; lane 7, 10-fold diluted; lane 8, 100-fold diluted; lane 9, 1000-fold diluted; lane 10, 10000-fold diluted cDNA.

seen after stimulation with both donor and third-party antigens. However, the relative amount of IL-2 and IL-4 mRNA produced by GIL was higher after donor-specific stimulation than after stimulation with third-party cells (P=0.02). Moreover, the relative amount of donor-specific IL-2 or IL-4 mRNA by these GIL cultures was clearly different. GIL cultures grown from endomyocardial biopsies taken during acute rejection synthesized significantly more donor-specific IL-2 mRNA than IL-4 mRNA (P=0.002). In two out of three cultures, we measured a 100-fold IL-2/IL-4 mRNA ratio.

IL-2 and IL-4 protein levels by GIL cultures

After donor-specific stimulation, the upregulated IL-2 mRNA expression resulted in measurable IL-2 protein levels from 4-8 h and accumulating at least 20 and 48 h (Table 3). In contrast, after challenge by third-party antigens the significant levels of IL-2 mRNA did not, or only at very low levels, result in subsequent IL-2 protein release by GIL (P<0.001, Table 3). After antigen-specific stimulation the maximum IL-2 protein production ranged between 28-849 pg/ml versus 0-55 pg/ml after third-party stimulation.

IL-4 protein levels were only measured in GIL culture I. In the supernatant of this culture, we found comparable IL-4 protein levels within 20-48 h after stimulation with donor or third-party antigens (Table 3).

Table 2 Ratio of IL-2 or IL-4/keratin mRNA expression in GIL cultures after stimulation with donor B-LCL and third-party control B-LCL.

	donor stimulation						third party stimulation					
	I[a]		II		III		I		II		III	
time (h)	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4
0	-[b]	-	-	-	-	-	-	-	-	-	-	-
1	1[c]	0.1	1	-	10	1	10	0.1	-	-	1	-
2	1	1	10	1	10	1	10	1	-	-	0.1	-
4	1	1	10	10	1000	10	1	0.1	-	1	1	0.1
8	1	1	1	1	10	10	1	0.1	1	-	0.1	1
12	1	0.1	10	1	1	10	1	1	1	1	0.1	0.1
20	100	0.1	10	-	0.1	0.1	0.1	1	10	-	0.1	0.1
48	100	-	10	-	1	-	-	-	1	-	1	1
72	0.1	-	-	-	-	-	-	-	0.1	-	-	-

[a]GIL culture number

[b]not detectable

[c]IL-2 and IL-4 are expressed as a ratio calculated from the titre of IL-2 or IL-4 mRNA product over the titre of amplified keratin mRNA product.

The amount of IL-2 mRNA and IL-4 mRNA were significantly higher after donor stimulation than after third-party stimulation (P=0.007 and P=0.02, respectively, Wilcoxon signed rank test). Donor-specific IL-2 mRNA titres were significantly higher than specific IL-4 mRNA titres (p<0.001).

Table 3 IL-2 and IL-4 protein production by GIL cultures after stimulation with donor B-LCL and third-party control B-LCL.

	donor stimulation						third party stimulation					
	I[a]		II		III		I		II		III	
time (h)	IL-2[b]	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4	IL-2	IL-4
0	<15[c]	<10	<15	NA[d]	<15	NA	<15	<10	<15	NA	<15	NA
1	<15	<10	<15		<15		<15	<10	<15		<15	
2	<15	<10	<15		<15		<15	<10	<15		<15	
4	62	<10	<15		70		48	<10	<15		<15	
8	115	<10	28		117		35	<10	<15		<15	
12	161	<10	35		136		43	<10	<15		<15	
20	849	16	24		131		50	<10	<15		<15	
48	524	15	29		224		55	21	<15		<15	
72	164	<10	20		125		35	<10	<15		<15	

[a]GIL culture number
[b]pg/ml
[c]Detection limit
[d]N.A., not available
The overall IL-2 protein was significantly higher after stimulation with donor than after third-party stimulation (P=0.002, Wilcoxon signed rank test).

Discussion

In the present study, we have studied the kinetics of IL-2 and IL-4 mRNA and subsequent protein production by *in vivo* primed GIL, grown from rejection biopsies, after *in vitro* stimulation with donor and third-party cells. The relative level of IL-2 mRNA transcription and subsequent IL-2 protein production was significantly higher after stimulation with donor antigens. This relative high level of IL-2 production by GIL cultures may reflect increased transcription in each cell or is the result of a higher frequency of donor-specific IL-2 producing T-cells in the graft during rejection. This explanation is in line with the results of Bishop et al. who showed a high frequency of IL-2 producing T-cells in sponge matrix allografts.[22] The results for IL-4 mRNA expression are in agreement with that of IL-2 mRNA. Also, at the mRNA level a donor-specific IL-4 signal was measured. However, during rejection, GIL cultures synthesized significantly more donor-specific IL-2 mRNA than IL-4 mRNA.
Cells cultured from the transplanted graft are stimulated *in vivo* by donor antigen on antigen-presenting cells, and as a consequence they are differentiated into either Th1 or Th2 cells and cannot be influenced by addition of cytokines as is the case for naive T-cells.[23] Upon restimulation *in vitro* these cells maintain a similar pattern of cytokine production. Moreover, both IL-2 (Th1) and IL-4 (Th2) producing cells proliferate in response to the added IL-2 *in vitro*.[24] Accordingly, it is likely that most of

the *in vitro* cytokine production is released by GIL that differentiated *in vivo* into either Th1 (IL-2) or Th2 (IL-4) producing lymphocytes.

Also after transplantation, the Th1/Th2 paradigm is frequently used to explain different immunological phenomena. Abundant production of Th1 cytokines is often accompanied with low secretion of Th2 cytokines and visa versa.[6,7]

Our results obtained in human lymphocytes at time of cardiac rejection are consistent with the hypothesis of the balance between IL-2 and IL-4 in allogeneic processes. Much of today's knowledge about the kinetics of cytokine production by lymphocytes is based on studies of clonal cells and peripheral blood cells after stimulation with either mitogens or alloantigens.[25-29] These studies show that IL-2 and IL-4 mRNA expression is rapidly but transiently induced after stimulation in T-cells. Also, in our bulk GIL cultures IL-2 and IL-4 mRNA transcription was detectable as early as 1-2 h after stimulation reached maximum levels between 2 and 48 h and base-line levels were approached by 20-72 h. Subsequent IL-2 protein release was detectable from 4 h with cytokines accumulating at least 20 and 48 h. In contrast to the IL-2 mRNA expression by mitogen-activated human T-cells as described by Shaw and colleagues[25] our mRNA profiles were definitely less spiking, which might be due to the composition of our bulk cultures. Phenotypic analysis showed that both $CD4^+$ and $CD8^+$ T-cells were present in the bulk GIL cultures. However, the time-course patterns of IL-2 and IL-4 production on the mRNA and protein level by $CD4^+$ and $CD8^+$ T-cells are only marginally different.[26] In contrast, the kinetics of cytokine mRNA production by lymphocytes is dependent on their activation level.[30]

Accordingly, we assume that in the graft, during acute rejection, cytokines are produced by allospecific T-cells with different stages of activation. After the initial priming, alloreactive T-cells home in the graft but nevertheless, represent, less than 1% of the total infiltrate.[31,32] Severe local inflammation, perhaps triggered by cytokine release might not only recruit T-cells with allospecificity to the graft but also T-cells with broader or irrelevant specificity. This mechanism might also explain why considerable levels of IL-2 and IL-4 mRNA expression were also found after stimulation with third-party cells.

In the present study, the time-course patterns of mRNA expression of IL-2 and IL-4 were comparable between GIL stimulated with donor cells and those activated with third-party cells, suggesting that also infiltrating T-cells with more public specificities underwent some level of activation. Nevertheless, these third-party cytokine mRNA levels, in contrast to donor-specific cytokine mRNA levels, did not regularly result in measurable protein levels.

A strong correlation has been shown between mRNA expression and protein production of various cytokines in GIL cultures isolated from rejected kidneys after activation with anti-CD3.[33] Anti-CD3 antibodies will activate all infiltrated T-cells including the donor-specific subpopulation. However, *in vivo* T-cell activation requires the interaction of antigen-presenting cells, peptide and a specific T-cell receptor.

Therefore, the results after *in vitro* stimulation with alloantigens is a better reflection of events within the allograft during acute rejection.
The discrepancy we found between mRNA and protein production after third-party but not donor-specific stimulation could of course be a detection phenomenon. Another explanation is that donor-specific signals are involved at the post-transcriptional level. GIL have T-cell receptors with low affinity for third-party antigens[34] and activation of these low affinity T-cell receptors might not lead to all the necessary signals required for translation of mRNA.
In summary, analysis of the kinetics of cytokine production showed that each individual GIL culture had its own characteristic mRNA and protein profile presumably due to differences in activation level of the alloreactive T-cells. In biopsy derived T-cell cultures, we measured a donor-specific mRNA signal for IL-2 and IL-4 but the relative IL-2 mRNA level was significantly higher resulting in abundant donor-specific IL-2 protein levels.

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Chapter 3.4

Different patterns in donor-specific production of T-helper 1 and 2 cytokines by cells infiltrating the rejecting cardiac allograft

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Abstract

Background: Cytokines play an important role in allograft rejection. The local production of cytokines by T-helper 1 and T-helper 2 cells within an allograft could influence the induction of graft rejection.

Methods: Therefore we studied the *in vitro* production of cytokines by cells infiltrating the graft. Graft-infiltrating cell cultures derived from human endomyocardial biopsy specimens more often produced interleukin-2 ($P<0.001$), interferon-γ ($P<0.001$), interleukin-4 ($P=0.02$) and interleukin-6 ($P=0.04$) after stimulation with a B-cell line obtained from the heart donor than after stimulation with a third-party B-cell line. Furthermore, the levels of these cytokines were significantly higher after donor stimulation than after third-party stimulation ($P<0.001$).

Results: Within the first 90 days after heart transplantation, significantly higher levels of interleukin-2 ($P=0.05$) and interferon-γ ($P=0.02$) were produced by donor-stimulated lymphocyte cultures derived from biopsy specimens taken during a rejection episode compared with cultures from biopsy specimens taken during a period without rejection. After 90 days, the levels of T-helper 1 cytokine (interleukin-2 and interferon-γ) production were, irrespective of the rejection grade, comparable with those found in the cultures from rejection biopsy specimens taken early after transplantation. With regard to T-helper 2 cytokines (interleukin-4 and interleukin-6), no relation was found with the presence of rejection at any time after transplantation.

Conclusions: These data suggest that in the first 3 months after heart transplantation, acute rejection is associated with the production of increased levels of T-helper 1 cytokines, but not of T-helper 2 cytokines by donor stimulated graft-infiltrating lymphocytes. Thereafter, the T-helper 1 cytokine production of graft-infiltrating cells remained high, suggesting a continuous state of immunologic activity even in the absence of rejection.

Introduction

Cytokines play a key role in the generation of alloreactive effector cells and therefore are important in allograft rejection. Two distinct cytokine secretion patterns have been defined in mouse $CD4^+$ T-cell clones.[1] T-helper 1 (Th1) cells produce mainly interleukin (IL)-2, IL-3, interferon (IFN)-γ, tumor necrosis factor-α and -ß, whereas T-helper 2 (Th2) cells secrete IL-3, IL-4, IL-5, IL-6 and IL-10. Romagnani[2] presented evidence for the existence of similar Th1 and Th2 subsets in man. Th1 cytokines favor the activation of cytotoxic lymphocytes and natural killer cells, whereas Th2 cytokines activate the humoral immune system and may be involved in the induction of non-responsiveness.[3] In efforts to monitor rejection, cytokines, their receptors or the corresponding mRNA were measured in peripheral blood or directly in endomyocardial biopsy specimens (EMBs). The levels of IL-2, IL-2 receptor and tumor necrosis factor-α in serum of peripheral blood[4,5] or coronary sinus blood[6] from cardiac allograft recipients did not correlate with the histologic grade of rejection in EMBs. In several studies cytokine gene expression was determined in cardiac allografts by polymerase chain reaction. IL-2, IFN-γ, tumor necrosis factor-ß and IL-4 mRNA were specifically expressed in mouse cardiac allografts and not in isografts or native hearts.[7,8] In EMBs from transplanted cynomolgus monkeys, IFN-γ mRNA was already found to be present before histologic evidence of rejection, whereas IL-1ß, IL-2 and IL-6 mRNA transcripts were expressed simultaneously with signs of moderate rejection.[9] After clinical heart transplantation (HTx), an increased expression of IL-2 mRNA was found during rejection,[10,11] whereas others reported a correlation of acute rejection with IL-6 and transforming growth factor-ß expression in EMB.[12,13] Cytokines can also be detected with the use of monoclonal antibodies and immunohistochemical techniques. In this way, the presence of IL-2 and IFN-γ located in lymphocytes of EMBs from cardiac allograft recipients were shown to be indicative for rejection, whereas IL-6 was rarely detected.[14]

However, with the previously mentioned techniques, it is impossible to show the exact cellular source of the cytokine expression or production or to quantify the levels of cytokines produced. In addition, when cytokine mRNA expression is present, protein production is not necessarily the result, whereas protein production of cytokines is always the consequence of mRNA expression.[15] Lymphocytes from the recipient that infiltrate the allograft (graft-infiltrating lymphocytes: GIL) and can be propagated from EMBs taken after HTx[16,17] are the relevant cells to study cytokine production profiles in relation to cardiac allograft rejection. These GIL contain, amongst others, donor-specific cytotoxic T-cells, which play an important role in acute rejection.[18-20] The activation of these cells can be influenced by the local secretion of cytokines. Only two groups studied cytokine production by GIL. Kirk et al.[21] measured IL-2, IL-4, IFN-γ, and tumor necrosis factor-α production in nine donor stimulated GIL cultures derived from renal biopsy specimens, and showed that only

IL-2 distinguished reversible from irreversible rejecting allografts. The second study described detectable frequencies of GIL secreting IFN-γ, IL-6, and IL-10 in isolated cells again from irreversible rejected kidneys, whereas cells from non-transplanted kidneys showed either low or undetectable expression of these cytokines.[22] Both studies investigated small numbers of GIL, without mentioning differences between nonrejection and rejection biopsy specimens or between donor and third-party reactivity. In the present study, a large panel of GIL cultures (n=49) derived from EMBs taken after HTx were analyzed with respect to the presence of donor-specific Th1-like and Th2-like cells. In addition, we studied whether different levels in IL-2, IFN-γ, IL-4 and IL-6 production were correlated with acute rejection at different time points after HTx.

Patients and methods

We studied 49 GIL cultures propagated from EMBs of 30 heart transplant recipients. Most acute rejections occurred during the first 90 days after HTx.[23] GIL growth was positively correlated with increasing histologic rejection grade, and most successful growth was shown in the first 3 postoperative months.[17] Twenty EMBs were taken in the first 90 days after HTx, and 29 EMBs were taken more than 90 days after HTx.

Grading of the EMB was according to the guidelines of the International Society of Heart and Lung Transplantation.[24] We divided the EMBs into two groups: (1) those without infiltrates or with infiltrates, but without myocyte damage: nonrejection EMB (grades 0 and 1; *n*=26) and (2) those with infiltrates and myocyte damage: rejection EMB (grades 2 and 3; *n*=23).

Cultures

GIL were cultured from EMBs in 96-wells U-bottom tissue culture plates (Costar, Cambridge, Mass.) as described before[17] in culture medium (Roswell Park Memorial Institute medium-1640 Dutch Modification; Gibco, Paisley, Scotland) supplemented with 10% pooled human heat-inactivated serum, 4 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) in the presence of approximately 30 U/ml exogenous IL-2 (lectin-free lymphocult T-LF, Biotest AG, Dreieich, Germany) and $1x10^5$ irradiated (30 Gy) autologous peripheral blood mononuclear cells. Only if cell growth was slowing down or cell death was observed (27 cultures) were the cultures restimulated once with a third-party Epstein-Barr virus-transformed B-cell line.[25] This cell line shared no human leukocyte antigen (HLA) with the donor or with the third-party used in the stimulation experiments to be described. No differences in cytokine production pattern were found in cultures which were or were not restimulated (data not shown). The mean culture period before testing was 34±14 days. The majority of the GIL cultures consisted exclusively of T-cells carrying the T-cell receptor α/ß (95% WT31 positive cells), the remaining 5% were NK-cells (CD16 positive cells), and macrophages and B-cells were not detected.

Cytokine production

GIL cultures do not produce cytokines continuously after *in vivo* stimulation with donor antigen; therefore, they have to be restimulated with this antigen *in vitro*. This restimulation was done with an irradiated B-cell line (60 Gy) of donor origin.

Before testing, the cultures were washed extensively and $5x10^4$ cells per well were incubated in eight replicates in a 96-wells V-bottom plate (Sterilin Limited, Hounslow, United Kingdom) at 37 °C in a humified atmosphere of 5% CO_2 in air during 24 hours in the absence of exogenous IL-2. Thereafter $5x10^4$ irradiated and washed cells of a B-cell line derived from the heart donor were added per well (total volume: 200 µl per well culture medium). A third-party B-cell line was used to test the donor

specificity of the response. After 20 hours of co-culture, 150 µl supernatant per well was harvested and the concentrations of IL-2, IFN-γ, IL-4 and IL-6 were determined with enzyme-linked immunosorbent assay (ELISA). Unstimulated cell cultures and the B-cell line alone served as controls.

Enzyme-linked immunosorbent assay
The concentration of cytokines in the supernatants of the GIL cultures was measured with the following ELISA-kits: IL-2 (Immunotech, Marseille, France) detection range 15 to 1000 pg/ml IL-2; IFN-γ (Life Technology, Breda, the Netherlands) detection range 80 to 5000 pg/ml IFN-γ; IL-4 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands) detection range 10 to 450 pg/ml IL-4; IL-6 (CLB, Amsterdam, the Netherlands) detection range 7 to 450 pg/ml IL-6. Values higher than three standard deviations above the lower detection limit were considered positive.

Results

Cytokine production in GIL cultures and donor specificity

Cytokine production was measured in 49 GIL cultures derived from EMB of 30 heart transplant recipients. Unstimulated cells did not produce IL-2, IFN-γ, IL-4, or IL-6. Also, stimulator cells alone did not produce IL-2, IFN-γ, or IL-4. In a few cases (13%), stimulator cells alone produced IL-6 (median 24, range 7 to 290 pg/ml), and this production was subtracted from the IL-6 production of the stimulated GIL cultures.
Approximately half (25 of 49; 51%) the GIL stimulated with donor cells produced Th1 (IL-2, IFN-γ, or both) as well as Th2-type (IL-4, IL6, or both) cytokines. Th1-like cytokines only were produced by 33% (16 of 49) of the cultures, whereas 6% (3 of 49) produced Th2-like cytokines only. In some culture supernatants no detectable cytokine levels could be measured (5 of 49; 10%). Thus, after stimulation with donor antigens Th1 cytokines were produced significantly more often than Th2 cytokines (41 of 49 versus 28 of 49; P=0.004, chi-squared test).
IL-2 and IFN-γ were found in 78% and 73% of the supernatants, respectively, whereas IL-4 and IL-6 were only detected in 35% and 43% of the cultures, respectively (Table 1). Significantly less GIL cultures produced cytokines after third-party induction than after donor stimulation. Not only the number of cytokine-producing cultures proved to be higher after donor than after third-party stimulation, but the cytokine production of the individual cell cultures also was higher after donor-specific stimulation (Figure 1: *A*, IL-2; *B*, IFN-γ; *C*, IL-4; *D*, IL-6).
No correlation was found between Th1 or Th2 profiles and the phenotypic composition (CD4, CD8, CD16 and WT31) of the cultures or the culture period (data not shown).

Cytokine production in relation to acute rejection

Most acute rejections occurred during the first 90 days after HTx.[23] We tested 20 GIL cultures derived from EMBs taken during this early posttransplant period: 8 from

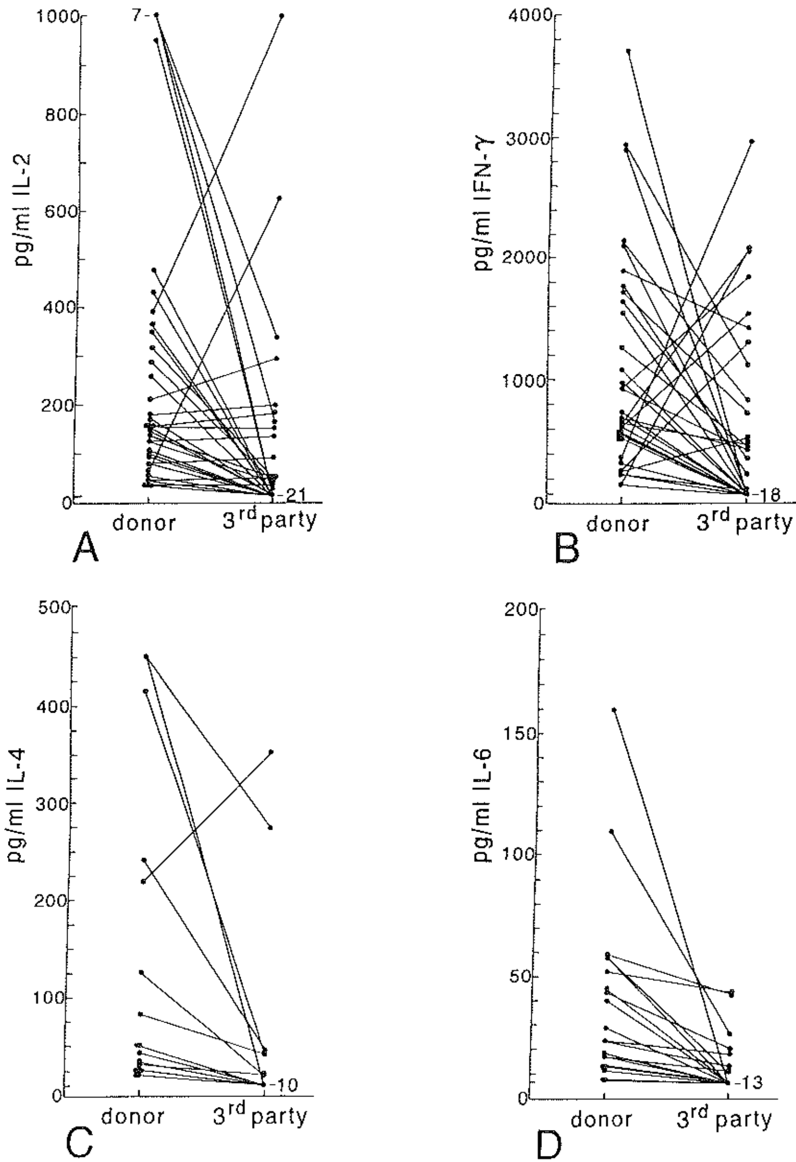

Figure 1 Graft-infiltrating lymphocytes that produced detectable levels of cytokines after stimulation were compared after stimulation with donor B-cell lines (B-LCL) or with third-party B-LCL. More interleukin (*IL*)-2 (**A**, P<0.001), interferon (*IFN*)-γ (**B**, P<0,001), IL-4 (**C**, P<0,001) and IL-6 (**D**, P<0,001) were produced after donor stimulation (both one-tailed paired sample t-test after logarithmic transformation and sign test). In cases of coinciding values the number of observations is indicated.

nonrejection and 12 from rejection EMBs, and 29 cultures from EMBs taken more than 90 days after transplantation, of which 18 were from nonrejection and 11 from rejection EMB.

In the first 90 days after HTx significantly more IL-2 was produced after donor stimulation of the GIL cultures derived from EMBs with signs of rejection (median 154 pg/ml IL-2, range 15 to 1000 pg/ml) compared with those from EMBs without signs of rejection (median 42 pg/ml IL-2, range 15 to 359) (P=0.05; Figure 2, *A*). Comparable results were obtained for IFN-γ during this early period (Figure 2, *B*). Cultures from rejection EMBs produced 659 pg/ml IFN-γ (range 80 to 2898) which was significantly different from nonrejection EMBs (median 176 pg/ml IFN-γ, range 80 to 1787) (P=0.02). In contrast, both groups of EMBs produced comparable levels of IL-4 and IL-6 after donor-specific stimulation (Figure 3: *A*, IL-4; *B*, IL-6). In addition, no difference in cytokine production was found when we compared (within the group of cultures from nonrejection EMBs taken early after HTx) EMB without infiltrates (grade 0) with EMB with infiltrates but no myocyte damage (grade 1) (data not shown).

For EMBs taken more than 90 days after HTx no differences in production for both Th1 cytokines (Figure 2: *C*, IL-2; *D*, IFN-γ) and Th2 cytokines (Figure 3: *C*, IL-4; *D*, IL-6) were found between cultures from rejection and nonrejection EMBs.

Table 1 Graft-infiltrating lymphocyte cultures (*n*=49) with detectable levels of cytokine production after stimulation either with a donor B-cell line (B-LCL) or with a third-party B-LCL.

	Cytokines produced			
Stimulator	IL-2	IFN-γ	IL-4	IL-6
donor	78% (38/49)	73% (36/49)	35% (17/49)	43% (21/49)
3rd party	38%[a] (18/48)	38%[b] (18/48)	14%[c] (7/48)	22%[d] (11/48)

IL, Interleukin; *IFN*, interferon.
Statistical analysis (see footnotes) was donor response compared with third-party response for each cytokine (chi-square test)
[a]P<0.001, [b]P<0.001, [c]P=0.02, [d]P=0.04

Discussion

In GIL cultures derived from EMBs, the relation of donor-specific cytotoxic T-cells and acute rejection has been studied extensively.[17-20,26] Although the importance of cytokine production within the transplanted heart for the induction of rejection has been stressed by several investigators,[6-14,27] only two clinical studies on this subject are available and both investigated kidney transplants with irreversible rejection.[21,22]

Figure 2 Production of T-helper 1 cytokines after donor stimulation in graft-infiltrating lymphocyte cultures derived from rejection and nonrejection endomyocardial biopsy specimens (EMBs) taken during the first 90 days after heart transplantation (**A**, n=12, rejection EMBs; **B**; n=8, nonrejection EMBs) or more than 90 days after heart transplantation (**C**, n=11, rejection EMBs; **D**; n=18, nonrejection EMBs). During the first 90 days after heart transplantation, more interleukin (*IL*)-2 (P=0.05) and interferon (*IFN*)-γ (P=0.02) were produced by graft-infiltrating lymphocytes from rejection than from nonrejection EMBs (Mann Whitney U test); later, after heart transplantation, the levels were comparable (IL-2, P=0.47; IFN-γ, P=0.29).

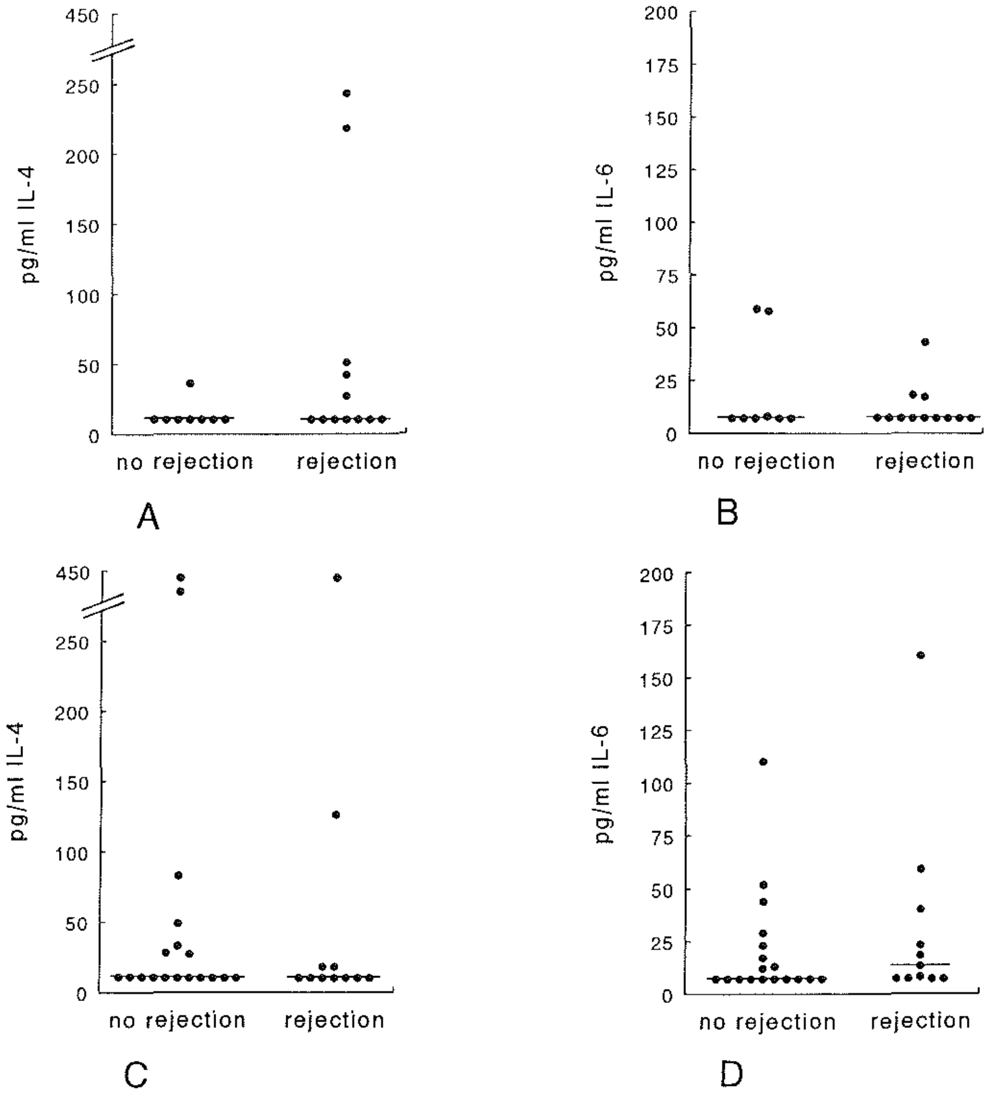

Figure 3 Production of T-helper 2 cytokines after donor stimulation in the same cultures as Figure 2 during the first 90 days after heart transplantation (**A** and **B**) and more than 90 days after heart transplantation (**C** and **D**). No significant difference between rejection and nonrejection endomyocardial biopsy specimens in interleukin (*IL*)-4 and IL-6 production up to 90 days after heart transplantation (P=0.08 and P=0.25, respectively) and more than 90 days (IL-4, P=0.41; IL-6, P=0.19) after heart transplantation.

The present study reports on the donor-specific cytokine production of GIL cultures and the relation with acute rejection.
Both Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-6) were produced significantly more often and at higher levels after donor stimulation than after third-party stimulation (Table 1 and Figure 1). Only a few cultures resulted in a higher cytokine response after third-party stimulation, which could be due to Epstein-Barr virus-specific response or to the fact that patients could have received a pretransplant blood transfusion sharing HLA antigens with the third-party stimulator. The production of relative high levels of cytokines within a short time (20 hours) after stimulation with donor antigens indicates that the GIL were primed (memory) cells.[28] The possibility of influencing the cytokine profile during a secondary immune response remains elusive (reviewed by O'Garra);[29] however, cytokines added *in vitro* during the development of Th1 and Th2 clones can determine their cytokine profile.[30] In contrast, cells which had already differentiated into Th1 or Th2 cells use IL-2 or IL-4, respectively, as autocrine growth factor. In addition, because both Th1 and Th2 cells proliferate in response to IL-2 added *in vitro*,[31] it is likely that the cytokine production pattern of *in vivo* primed GIL cultures reflects the original situation in the graft. Although Th1 cells seemed to retain their responsiveness to IL-2 longer than Th2 cells,[31] which might result in more cells secreting Th1 cytokines in the GIL cultures, a similar predominance of Th1 cytokines within the graft was found after murine heart transplantation.[27]
In our heart transplant program, as in other centers, most acute rejections occur in the first 90 days after HTx.[23] Although an overlap in cytokine levels was detected between rejection and nonrejection samples, it is obvious that in this period more IL-2 (Figure 2, *A*) and IFN-γ (Figure 2, *B*) were produced by cultures derived from EMBs with signs of rejection compared with those without signs of rejection. Previously a positive strong correlation was also found after clinical HTx between IL-2 mRNA expression and rejection in EMB.[10,11] The present results on IL-2 protein production in GIL cultures are in agreement with these findings on the transcriptional level. The specific expression of IL-2 and increased expression of IFN-γ mRNA and protein found in allogeneic compared with syngeneic mouse heart transplants[7,8,27] and the correlation of mRNA expression of these cytokines with the severity of acute rejection episodes in transplanted non-human primate[9] and human[14] hearts are in harmony with our results on cytokine protein production level. All these studies confirm the importance of Th1 cytokines in acute rejection. Apart from stimulation of differentiation and proliferation of T-helper cells, IL-2 enhances the generation of cytotoxic T-cells,[32] which can recognize and kill allogeneic target cells. The increment of IL-2 in rejection EMBs could enhance the maturation of donor-specific cytotoxic T-cells in EMBs. The presence of a rather large amount of immature cytotoxic cells in GIL cultures from EMB[26] supports a role of locally produced cytokines in the final maturation step.

Furthermore, IFN-γ can exert multiple effects on other cells, including an upregulation of surface class II antigen expression.[33] Under normal circumstances, human vascular endothelium expresses HLA class I antigens but little or no HLA class II antigens.[34] However, the expression of the latter will increase during rejection[35] and is probably induced by the IFN-γ produced by GIL.
Although we found a positive relation for Th1 cytokines and rejection early after HTx, comparable increased IL-2 and IFN-γ levels were found both in GIL from early rejection EMBs and in GIL from EMBs taken late after transplantation. It is possible that, in the mean time, immunologic countermechanisms were induced, which would have resulted in nonresponsiveness despite the presence of Th1 cytokine-producing cells.
With regard to the Th2 (IL-4 and IL-6) cytokines, no relation was found with the presence of rejection at any time after transplantation (Figure 3). Others also did not find a predictive value of IL-4 mRNA expression in EMBs for acute rejection of human cardiac allografts.[10,12,13] One group observed an increased gene expression of IL-6 in EMBs during rejection.[12,13] However, IL-6 mRNA expression is frequently found in human heart tissue both before and after transplantation[10] and in native murine hearts or isografts.[8] A reason may be that IL-6 is secreted not only by activated Th2 cells[1] but also by activated macrophages[36] and endothelial cells,[37] suggesting a more overall expression of this cytokine in organs. In the present study only IL-6 production by GIL was determined, excluding the contribution of nonlymphoid cells; still, no relation with rejection was found. These results imply that Th2 cytokines may be less relevant in the early rejection process. Others suggested a role for both IL-2 and IL-4 during rejection, but this hypothesis was concluded from studies in nonimmunosuppressed mice bearing cardiac allografts.[7,8] The differences in IL-4 may be the result of immunosuppressive therapy applied in human beings.
In conclusion, the described study showed that in the first 3 months after HTx, the increased quantity of Th1 but not Th2 cytokines produced by GIL correlated with acute rejection. Thereafter, the Th1 cytokine production of GIL remained high, suggesting a continuous state of immunological activity even without histologic signs of rejection.

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Chapter 4

Intragraft monitoring of graft vascular disease

Chapter 4.1

Phenotypic analysis of lymphocytes infiltrating human cardiac allografts during the development of graft vascular disease

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Abstract

Graft vascular disease (GVD) is mediated, at least in part, by cellular processes. Therefore, we cultured graft-infiltrating lymphocytes (GIL) from endomyocardial biopsies (EMB) taken during the first year after transplantation, determined their phenotypic composition, and correlated it to GVD. No difference was found between patients with and without GVD one year after transplantation. $CD4^+$ cells were always predominant present to $CD8^+$ cells, and no difference in phenotypic composition was found between EMB derived from patients with or without signs of GVD.

In conclusion, the development of GVD is not associated with AR, GIL growth from EMB or their phenotypic composition.

Introduction

In several studies, we and others have shown that alloactivated graft-infiltrating lymphocytes (GIL) can be expanded *in vitro* from endomyocardial biopsies (EMB) in IL-2-containing medium.[1-3] This culture system is thought to promote growth from *in vivo* activated GIL only.[3] We also have suggested that the outgrowth of GIL from EMB and their phenotypic composition corresponded with histological findings of acute rejection (AR)[1] and graft vascular disease (GVD).[2] In the present study, we applied the same method of culturing GIL and studied per patient the proportion of EMB from which GIL could be obtained and their phenotype in 3 periods during the first year after heart transplantation (HTx) in an attempt to show whether our previous findings in groups of patients could be applied for individual cardiac transplant recipients. We compared these parameters for patients with and without GVD in the first year after HTx.

Patients and methods

We studied 60 cardiac allograft recipients, who were transplanted between March 1991 and September 1993. EMB were taken at regular intervals after HTx and examined histologically. According to the criteria of the International Society of Heart and Lung Transplantation,[4] AR was defined as cell infiltrate with myocyte necrosis, and anti-rejection therapy was prescribed. Because most AR episodes occur in the first 3 months after HTx,[5] we focussed on this period.
One year after HTx the diagnosis of GVD was assessed by coronary angiography and scored by one of us (AHMM B). GVD was defined as clinically significant vascular changes including minimal wall irregularities.[6] Twenty of the studied patients did have signs of GVD in their angiography one year after HTx, and 40 patients did not.

Culture method and phenotypic analysis
We received EMB from 49 out of 60 patients taken in the first year after HTx to obtain GIL. GIL were cultured from EMB in IL-2 containing medium as described before.[1,2] GIL were analyzed by two-colour flow cytometry on a FACScan for the expression of cell surface markers. Initial screening was performed with the combinations WT31 FITC-CD3 PE, CD4 FITC-CD8 PE, and CD16 FITC-CD56 PE. When $CD3^{+}WT31^{-}$ cells were found, the GIL cultures were stained for T-cells bearing TCR-γδ. WT31 (TCR-αß) and TCR-γδ were obtained from Becton Dickinson (San Jose, CA, USA) and the other monoclonal antibodies from Immunotech (Marseille, France).

Statistics
We determined from all individual patients the fraction of EMB in the first year post transplantation showing signs of AR, the GIL growth from EMB, and their phenotypic composition in fixed periods after HTx. The Mann-Whitney U test was used to evaluate the differences between the patient groups.

Results

We found no correlation between the incidence of AR and GVD (Table 1). The development of GVD in the first year after HTx did not influence the cell growth from EMB (Table 2). No relation was detected in the mean percentage TCR-αß, TCR-γδ, CD3, CD16, CD4 and CD8 at any time after HTx (Table 3). More $CD4^+$ than $CD8^+$ cells were found and this proved to be independent of GVD development.

Table 1 Percentage of endomyocardial biopsies with signs of rejection during different periods after transplantation in patients without (n=40) and patients with (n=20) signs of graft vascular disease (GVD) one year after heart transplantation (HTx).

months after HTx	without GVD (mean %)[a]	with GVD (mean %)[a]	P-value
0-3	25	26	0.75
3-6	20	17	0.68
6-12	9	8	0.70

[a]mean value of all individual patients

Table 2 Proportion of graft-infiltrating lymphocyte growth from endomyocardial biopsies during different periods after transplantation in patients without (n=30) and patients with (n=18) signs of graft vascular disease (GVD) one year after heart transplantation (HTx).

months after HTx	without GVD (mean %)[a]	with GVD (mean %)[a]	P-value
0-3	43	50	0.30
3-6	32	48	0.20
6-12	28	30	0.67

[a]mean value of all individual patients

Discussion

In agreement with the previous study[1], in this report, we found CD4 to be the predominant phenotype in most cultures at any time after transplantation.
Since GVD was diagnosed by annual angiography, the exact moment of onset of

Table 3 Mean phenotypic composition of cell growth from endomyocardial biopsies during different periods after transplantation in patients without and patients with signs of graft vascular disease (GVD) one year after heart transplantation (HTx).

	months after HTx	without GVD (mean %)[a]	with GVD (mean %)[a]	P-value
TCR-αß	0-3	90	93	0.54
	3-6	97	94	0.38
	6-12	79	88	0.47
TCR-γδ	0-3	1.9	1.6	0.73
	3-6	0.1	0.7	0.66
	6-12	7.4	1.7	0.98
CD3	0-3	93	94	0.68
	3-6	97	95	0.30
	6-12	88	90	0.77
CD16	0-3	7.2	4.3	0.37
	3-6	1.5	4.0	0.60
	6-12	11.3	7.6	0.95
CD4	0-3	54[b]	60[e]	0.46
	3-6	68[c]	66[f]	0.98
	6-12	63[d]	53[g]	0.45
CD8	0-3	35[b]	31[e]	0.53
	3-6	31[c]	33[f]	1.0
	6-12	20[d]	29[g]	0.34

[a]Mean value of all individual patients
[b,c,d,e,f,g]Mean percentage CD4 versus CD8 in cultures derived from EMB of patients without signs of GVD taken 0-3 ($P<0.001$)[b], 3-6 ($P=0.003$)[c] and 6-12 ($P<0.001$)[d] months after HTx and from EMB of patients with signs of GVD taken 0-3 ($P=0.002$)[e], 3-6 ($P=0.01$)[f] and 6-12 ($P=0.02$)[g] months after HTx.

GVD could not be determined. Therefore, we analyzed several parameters after different periods in the first year after HTx and their relationship with GVD. We could not detect any correlation with the development of GVD during the first year after HTx and the number of EMB with signs of AR, growth patterns of GIL or their phenotype. Recently, we published that in a different population of cardiac transplant recipients, patients with GVD had a higher percentage of $CD8^+$ T-lymphocytes during the first year after transplantation.[2] The discrepancy between this and the previous

report is the difference in analysis: the present study per individual patient and the previous study per GIL culture.

From the described study, we conclude that GVD is not related to AR, and also the GIL growth and their phenotype could not make a distinction between patients who did or did not develop GVD in the first year after transplantation.

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Chapter 4.2

Donor-specific cytokine production by graft-infiltrating lymphocytes induces and maintains graft vascular disease in human cardiac allografts

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Abstract

Backgroud: The development of graft vascular disease (GVD) in the allograft is a major impediment for long-term survival of heart transplant recipients. GVD may be mediated by cellular processes, in response to the transplanted heart, and regulated by cytokines.

Methods: We studied donor-specific cytokine production patterns in graft-infiltrating lymphocyte cultures propagated from endomyocardial biopsies. The biopsies were derived from patients with and without signs of GVD, as diagnosed by angiography at one year after heart transplantation.

Results: In the first year after transplantation, significantly more T-helper (Th) 1 cytokines (interleukin [IL]-2: P=0.04; interferon-γ: P=0.01), but not Th2 (IL-4 and IL-6) cytokines, were produced by cultures of patients with GVD compared with patients without GVD. Thereafter, the Th1 cytokine levels in patients with GVD normalized to the levels of patients without GVD. Detectable levels of IL-6 were produced significantly more often (P=0.009) by cultures obtained more than one year after transplantation from patients with GVD.

Conclusions: The results suggest that high levels of Th1 cytokines produced by graft-infiltrating lymphocytes early after transplantation may be responsible for activation of vascular endothelium, leading to migration and proliferation of smooth muscle cells that is characteristic of GVD. IL-6, produced later after transplantation, continues this process by promoting smooth muscle cell proliferation.

Introduction

Graft vascular disease (GVD) is reported to be a major limiting factor for medium-and long-term human cardiac allograft survival.[1] In our transplant center, the incidence of GVD, as observed by coronary angiography, is 26% at one year after heart transplantation (HTx).

GVD is characterized by accelerated, diffuse, and concentric intimal proliferation, involving both the microvasculature and epicardial vessels in the heart.[2] Arterial lesions develop in donor vessels and are absent in recipient vessels, which suggests that GVD results from an immune-mediated process, initiated at and located within the donor vasculature. However, the pathogenesis of GVD remains poorly understood and may be multifactorial. The number of acute rejection episodes, the presence of anti-HLA antibodies, disorders of lipoprotein metabolism, the influence of immunosuppressive drugs, and cytomegalovirus infection may all be involved (reviewed by Tullius).[3] In our transplant center, GVD could not be correlated to ischemic heart disease before transplantation, preoperative reactivity against a panel of lymphocytes, gender, age, acute rejection, hypertension, serum cholesterol and triglycerides, use of calcium antagonists, or cytomegalovirus infection.[4]

A leading hypothesis is that GVD arises from chronic immune stimulation of the donor endothelium.[5,6] According to this view, local cytokine and growth factor production induces activation of the endothelium, which in turn results in neointimal thickening and vascular smooth muscular remodelling.[2,7] However, there is little clinical evidence to support this explanation, which links GVD with specific cytokines affecting the endothelium.

Recently, we have demonstrated that graft-infiltrating lymphocytes (GIL), propagated from endomyocardial biopsies (EMB) taken during acute rejection after HTx, can produce significant levels of T-helper (Th) 1-like cytokines (interleukin [IL]-2 and interferon [IFN]-γ) after donor-specific stimulation of these GIL.[8] To clarify the significance of cytokines produced by lymphocytes that infiltrate the allograft, we studied whether there is a relationship between Th1- and Th2-like (IL-4 and IL-6) cytokines produced by GIL and the development of GVD. GIL cultures derived from EMB taken after HTx were analyzed with respect to the presence of donor-specific Th1- and Th2-like cells. In addition, we studied whether the levels of IL-2, IFN-γ, IL-4 and IL-6 production were correlated with the development of GVD. Because the development of GVD in the first year after HTx seems crucial for the course of the disease in the subsequent years,[4] we determined the cytokine pattern of the GIL at different time points after HTx, focussing especially on the first postoperative year.

Materials and methods

Patients

All patients received one preoperative blood transfusion. Cyclosporine and low-dose prednisone were given only as maintenance immunosuppression. EMB were routinely taken at weekly intervals during the first 6 weeks after HTx, and once every 2 weeks during the following 4 weeks. Later, EMB were taken less frequently, declining to once every 4 months at one year.

One year after HTx, GVD was visually assessed by coronary angiography and scored by one of the authors (AHMM B). She was unaware of the cytokine data when she was analyzing the angiograms. GVD was defined as all vascular wall changes, including minimal wall irregularities.[4]

Before and after the diagnosis of GVD, 85 fresh (noncryopreserved) GIL cultures were propagated from EMB of 27 patients without GVD and of 15 patients with signs of GVD at one year after HTx. We arranged these cultures according to successive time intervals after HTx (Table 1). The group of patients without and with GVD did not differ significantly in the number of patients who did or did not reject their allograft in their first postoperative year (without GVD: 2 nonrejectors, 25 rejectors; with GVD: 4 nonrejectors, 11 rejectors) nor in the number of acute rejections (without GVD: 3.4±3.0; with GVD: 3.9±2.8 [mean±SD]). Also, no differences in the number of acute rejection EMB was found between the cultures derived from patients without GVD and those with GVD in the different time periods (0-3 months after HTx: without GVD, 9 cultures were derived from EMB without acute rejection and 9 cultures were from EMB with acute rejection; with GVD, 5 and 7 cultures were derived, respectively; 3-6 months after HTx: without GVD, 8 and 4 were derived, respectively; with GVD, 4 and 5 cultures were derived, respectively; 6-12 months after HTx: without GVD, 5 and 1 cultures were derived, respectively; with GVD, 4 and 2 cultures were derived, respectively; >12 months after HTx: without GVD, 10 and 2 were derived, respectively; with GVD, 6 and 4 cultures were derived, respectively). When more than one GIL culture was determined for any patient, the mean cytokine production was calculated.

Table 1 Number of graft-infiltrating lymphocyte cultures tested per number of patients without graft vascular disease (GVD) and with signs of GVD per period after heart transplantation (HTx).

	GVD-		GVD+	
Months after HTx	No. of patients	No. of cultures	No. of patients	No. of cultures
0-3	11	18	7	12
3-6	6	12	4	9
6-12	5	6	5	6
0-12	17	36	11	27
>12	11	12	6	10

Cultures

GIL were cultured from EMB in 96-well U-bottom tissue culture plates (Costar, Cambridge, MA), as described previously,[8] in culture medium (RPMI-1640 Dutch Modification [Life Technologies, Paisley, Scotland], supplemented with 10% pooled human heat-inactivated serum, 4 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin). Culture was performed in the presence of approximately 30 U/ml exogenous IL-2 (lectin-free lymphocult T-LF; Biotest AG, Dreieich, Germany) and 10^5 irradiated (30 Gy) autologous peripheral blood mononuclear cells per well. If cell growth was slowing

down or cell death was observed (58% of the cultures), before sufficient cell numbers were reached, the cultures were restimulated once with a third-party Epstein Barr virus-transformed B-cell line (B-LCL) ($5x10^3$/well).[9] This cell line shared no HLA antigens with the donor nor with the third-party cell line used in the stimulation experiments described below. The mean culture period before testing was 33 days (SD: 13 days). The GIL cultures were analyzed by two- and three-color flow cytometry on a FACScan (Becton and Dickinson, San Jose, CA) for the expression of cell-surface markers. Sreening was performed with the combinations WT31/fluorescein isothiocyanate (T-cell receptor-αß), CD4/phycoerythrin, CD8/PERCP, and CD3/fluorescein isothiocyanate, CD16+56/phycoerythrin (natural killer cells).

Cytokine production

Before testing, the cultures were washed extensively and $5x10^4$ cells per well were incubated 8-fold in a 96-well V-bottom plate (Greiner, Alphen aan de Rijn, the Netherlands). Incubation was performed at 37°C in a humified atmosphere of 5% CO_2 in air during 24 hours, in the absence of exogenous IL-2. Thereafter, $5x10^4$ irradiated (60 Gy), and subsequently washed, B-LCL derived from the heart donor were added per well (total volume: 200 µl per well culture medium in the absence of IL-2). A third-party B-LCL was used to test the donor-specificity of the response. After 20 hours of co-culture, 150 µl of supernatant per well was harvested and the concentrations of IL-2, IFN-γ, IL-4, and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA). Unstimulated cell cultures and B-LCL alone served as controls.

ELISA

The concentration of cytokines in the supernatants of the GIL cultures was measured with the following ELISA kits: IL-2 (Immunotech, Marseille, France), detection range 15-1000 pg/ml IL-2; IFN-γ (kindly provided by Dr. P.H. van der Meide, Biomedical Primate Research Centre, Rijswijk, the Netherlands), detection range 80-5000 pg/ml IFN-γ; IL-4 (Central Laboratory of the Netherlands Red Cross-Blood Transfusion Service (CLB), Amsterdam, the Netherlands), detection range 10-450 pg/ml IL-4; IL-6 (CLB, Amsterdam, the Netherlands), detection range 7-450 pg/ml IL-6.

Statistics

For the statistical analysis of differences between patient groups, the two-sided Mann-Whitney U test was used (Tables 2 and 3; Figure 1).

Results

GIL cultures

The majority of the cultures from patients without GVD (mean: 95±8%) and with GVD (mean: 93±12%) consisted of T-cells carrying the T-cell receptor α/ß, the remaining cells were natural killer cells (6±17% and 6±12%, respectively). We could also not differentiate for CD4- and CD8-positive cells and GVD (CD4: without GVD, 61±31%, with GVD, 53±28%; CD8: without GVD, 31±31%, with GVD, 39±29%). Macrophages and B-cells were not present in the cultures.

Cytokine production in GIL cultures

Unstimulated GIL cultures did not produce IL-2, IFN-γ, IL-4, or IL-6. Stimulator B-LCL cells alone did not produce IL-2, IFN-γ, or IL-4 either, whereas IL-6 was produced by

B-LCL alone in 28% of the cases (mean: 38±57 pg/ml). This base level production was subtracted from the IL-6 produced by the stimulated GIL cultures.
Recently, we have demonstrated that a donor-specific cytokine production can be determined by stimulating the GIL cultures with donor B-LCL, because both Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-6) were produced significantly more often and at higher levels after donor stimulation than after third party stimulation.[8]

Cytokines produced before and after the diagnosis of GVD

We determined prospectively the cytokine production of donor-stimulated GIL cultures in the first year after HTx, before the diagnosis of GVD (Figure 1). During this period, significant more IL-2 (without GVD: median 140 pg/ml, range 15-469; with GVD: median 411 pg/ml, range 15-1000; P=0.04) and IFN-γ (without GVD: median 386 pg/ml, range 80-2913; with GVD: median 1197 pg/ml, range 80-5000; P=0.01) was produced by the cultures derived from patients that had GVD at one year after HTx, compared with the cultures from patients without GVD. However, after the first preoperative year, no difference between the patient groups were found (IL-2: without GVD, median 156 pg/ml, range 15-1000; with GVD, median 136.5 pg/ml, range 15-258; IFN-γ: without GVD, median 502 pg/ml, range 80-1509; with GVD, median 365.5 pg/ml, range 80-2520). Comparing the first year after HTx with the period thereafter, comparable levels of cytokines were produced by the donor-stimulated cultures from the patients without GVD. However, GIL cultures derived from patients with signs of GVD in their angiogram produced significantly more IL-2 (P=0.05) and IFN-γ (P=0.06) in the first year after HTx than in the period thereafter. The levels produced by GIL from EMB taken more than one year after HTx of the patients with signs of GVD were in the range of the group without GVD.
No differences for the Th2 cytokines IL-4 and IL-6 were detected in the first year after HTx (Table 2). After the diagnosis of GVD at one year after HTx, more IL-6 was produced by the cultures derived from patients with GVD, compared with those without GVD (P=0.007). This was caused by the higher frequency of cultures from patients with GVD (6/6: 100%) than without GVD (3/11: 27%) that produced detectable levels of IL-6 (P=0.009: Fischer's exact test, two sided P-value).

Cytokine production in the first year after transplantation

To determine the cytokine profile of GIL obtained at different time periods in the first preoperative year after transplantation, we divided this year in three consecutive periods: 0-3, 3-6 and 6-12 months after HTx (Table 3).
During the first 3 preoperative months, a trend (P=0.15) toward more IL-2 production by the cultures derived from patients with GVD compared with those without GVD was seen (Table 3A). This was accompanied by higher levels of IFN-γ production by the cultures derived from patients with GVD (P=0.10; Table 3A). Over the next 3

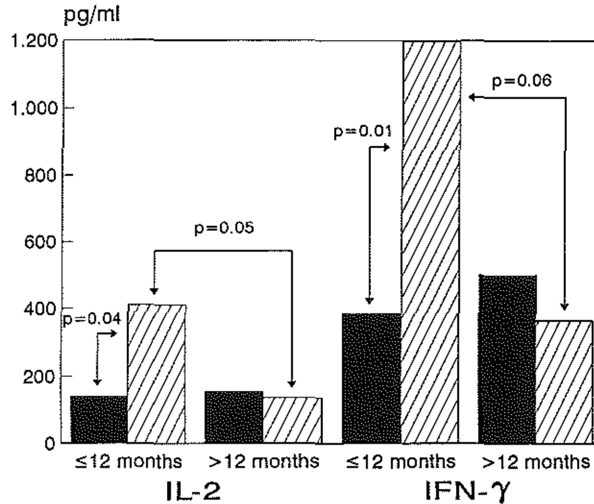

Figure 1 Median levels of Th1 (IL-2, IFN-γ) cytokine production after donor stimulation of graft-infiltrating lymphocyte cultures derived from endomyocardial biopsies taken before (0-12 months) and after (>12 months after heart transplantation (HTx)) the diagnosis graft vascular disease (GVD).
■, Cultures from patients without GVD; ▨, cultures from patients with GVD.

Table 2 Median levels of Th2 (IL-4 and IL-6) cytokine production after donor stimulation of graft-infiltrating lymphocyte cultures derived from endomyocardial biopsies taken before (0-12 months) and after (>12 months after heart transplantation (HTx)) the diagnosis of graft vascular disease (GVD).

	IL-4 (pg/ml)			IL-6 (pg/ml)		
Months after HTx	GVD-	GVD+	P[2]	GVD-	GVD+	P
0-12[3]	10[1] 10-160	26 10-149	0.20	11 7-110	14 7-138	0.26
>12[3]	10 10-450	50 10-450	0.35	7 7-32	14.5 11-34	0.007
P[4]	0.77	0.81		0.13	0.53	

[1]Data are presented as the median cytokine production per group of patients, with their range (minimum-maximum).
[2]Cytokine production from cultures of patients without GVD were compared with cultures of patients with GVD.
[3]Seventeen patients without and 11 patients with GVD were tested in the first year after transplantation, and 11 patients without and 6 patients with GVD were tested after one year after transplantation.
[4]Cytokines produced in the first year compared with more than one year after transplantation for patients without and with GVD.

months, this trend of increased IFN-γ production continued, resulting in significant differences between the patient groups (P=0.01). Thereafter, in the period from 6 to 12 months after HTx, comparable levels were produced by both groups.

For IL-4, a slight increment (P=0.15) of cytokine production was seen only in the cultures derived from EMB taken 6-12 months after HTx of patients with GVD, in comparison with the cultures derived from patients without GVD (Table 3B). There was no differentiation in IL-6 levels between patients with and without GVD in any of the three periods of the first year after HTx (Table 3B).

Table 3 Median cytokine production levels of the Th1 (A: IL-2 and IFN-γ) and Th2 cytokines (B: IL-4 and IL-6) after donor stimulation of GIL cultures derived from endomyocardial biopsies taken at different periods in the first year after heart transplantation (HTx) of patients without (GVD-) and with (GVD+) graft vascular disease.

A

Months after HTx GVD- (n)/GVD+ (n)	IL-2 (pg/ml)			IFN-γ (pg/ml)		
	GVD-	GVD+	P[2]	GVD-	GVD+	P
0-3 (11/7)	82 15-518[1]	266 15-552	0.15	271 80-1899	926 80-5000	0.10
3-6 (6/4)	195.5 33-877	201.5 100-1000	0.61	238.5 80-895	1644 1059-2224	0.01
6-12 (5/5)	149 15-348	257 15-1000	0.42	846 164-2913	526 242-5000	1.0

B

Months after HTx GVD- (n)/GVD+ (n)	IL-4 (pg/ml)			IL-6 (pg/ml)		
	GVD-	GVD+	P[2]	GVD-	GVD+	P
0-3 (11/7)	10 10-218[1]	20 10-149	0.25	7 7-44	9 7-229	0.43
3-6 (6/4)	10 10-125	21.5 10-213	0.35	16 7-53	15.5 12-31	0.91
6-12 (5/5)	83 10-450	10 10-77	0.15	57 7-160	14 7-59	0.55

[1]Data are presented as the median cytokine production per group of patients, with their range (minimum-maximum).

[2]Cytokine production from cultures of patients without GVD were compared with cultures of patients with GVD.

Discussion

The long-term success of organ transplantation is limited by the development of chronic rejection. The cause of GVD, or chronic rejection after HTx, may be endothelial cell activation, finally leading to intima proliferation, which is the characteristic of GVD. Tissue damage, e.g., by ischemia and reperfusion, induces early activation of endothelial cells, and causes expression of new adhesion molecules on surface of these cells. This is followed by macrophages infiltrating the graft and by secretion of monokines, which in turn results in another shift of adhesion molecules on endothelial cells. Such a process is an universal repair phenomenon in organ transplants.[10,11] GVD is clearly not the result of nonspecific endothelial activation alone; other factors must also play a role. Unique to the allograft is the subsequent infiltration of the graft by activated T-lymphocytes (GIL) and their production of cytokines when they encounter appropriate donor antigens. These allogeneic responses may be responsible for the characteristic vascular disease found after organ transplantation only. Therefore, we studied allogeneic immune processes within the transplanted heart in relation to the development of GVD. We have already demonstrated that neither growth frequency of GIL nor their phenotypic composition was differentiated between patients who developed GVD and those who remained free from GVD in the first year after HTx.[12] In this report, we measured the donor-specific cytokine production of GIL cultures and studied their relation with GVD.

An overlap in cytokine levels was detected between patients who did or did not have signs of GVD in their coronary angiogram at one year after HTx. However, it is obvious that more Th1 cytokines (IL-2 and IFN-γ), and not the Th2 cytokines IL-4 and IL-6, were produced by cultures derived from EMB with signs of GVD, compared with those without signs of GVD, in the first year after HTx. The overlap could be the result the method we used for detecting GVD. Rickenbacher and coworkers[13] already showed that 85% of cardiac transplant patients with normal angiograms had evidence of intima thickening by the intracoronary ultrasound method. Perhaps a clearer differentiation in cytokine production between the patient groups could be made by quantitatively determining the degree of intima thickening by such sensitive diagnostic techniques.

In animal studies, the importance of the Th1 cytokines has also been indicated. Tilney et al.[14] and Hancock et al.[15] showed in renal rat allografts that at the time when arteritis and glomerulitis appeared, 10-20% of cellular infiltrates, consisting of T-lymphocytes and macrophages, were positive for IL-2, IFN-γ, and tumour necrosis factor (TNF)-α in immunohistology. Russell et al.[16] also found early and sustained up-regulation of IFN-γ in mononuclear cells within the cardiac allograft in rats with chronic cardiac rejection. After human liver transplantation, Hayashi and coworkers[17] demonstrated more IL-2 and IFN-γ mRNA in explanted livers and biopsy samples

with the diagnosis of chronic rejection than in biopsies without evidence of rejection. However, Bishop et al.[18] could not detect any difference between Th1 cytokine mRNA expression in biopsy tissue derived from human liver allografts recipients with chronic rejection and normal liver tissue. In human kidney transplant recipients, Noronha et al.[19] could also not detect a correlation between chronic rejection and the expression of TNF-α and IFN-γ measured by immunocytochemical studies. Merville et al.[20] studied GIL grown from removed kidney allografts and found no difference in the frequency of IFN-γ-producing cells between patients with irreversible ongoing acute rejection and patients with chronic rejection complicated by superimposed acute rejection. So most clinical studies were not able to confirm the importance of Th1 cytokines in the development of GVD, which has been suggested by the studies in experimental animals.

What about the Th2 cytokines? Parallel to our results, Hayashi et al.[17] found no difference in the expression of IL-4 and IL-10 in liver allografts, whereas the IL-5 gene was significantly upregulated in the grafts with chronic rejection compared with stable grafts. In contrast, Bishop et al.[18] found significantly less IL-10 mRNA expression in human liver biopsies derived from patients with chronic rejection after transplantation, compared with normal livers. Also, Merville et al.[20] showed a lower frequency of IL-10-producing cells in GIL of removed human kidney allografts from patients with chronic rejection compared with patients with irreversible acute rejection.

It is difficult to compare the results mentioned above with our results. Although our results closely resemble of those found in the preclinical studies, they do differ from some of the clinical analyses. These differences could be the consequence of the organ transplanted, the immunosuppressive load, and the method used to diagnose GVD. Also, the methods used for detecting cytokines, and of course, the cellular source of the cytokines, can influence the results. Even more important could be the timing of the studies. In general, the investigators described only the cytokines found after the diagnosis of GVD was made. This is probably too late; the harm has already been done. Therefore, after our finding that Th lymphocytes play a role before the diagnosis of GVD, we longitudinally investigated in which period in the first postoperative year cytokines may play a crucial role in the development of GVD. A trend was detected toward more IL-2 and IFN-γ production in the first 3 postoperative months for the GIL cultures derived from patients with compared with those without GVD at one year after HTx. In fact, early IL-2 production stimulates the activation and proliferation of lymphocytes,[21] and IFN-γ recruits macrophages[23] into the graft and enhances cytotoxic T-lymphocyte differentiation[22-24] and MHC expression of the vascular endothelium.[25] The significance of IFN-γ was intensified 3-6 months after transplantation in the donor-stimulated cultures derived from patients with GVD. In the second half of the year after HTx, no difference in Th1 cytokine production was seen anymore, whereas in this period only slightly higher levels of IL-4 were

produced by the cultures of patients without GVD compared with those patients with GVD.

More than one year after HTx, no difference in Th1 cytokines between the patient groups was found by us, as the IL-2 and IFN-γ levels of the patients with GVD had decreased to the concentrations observed in the patients without GVD. The results emphasize the importance of measuring cytokine levels during the first year after transplantation, before the diagnosis of GVD.

The detectable production of IL-6 by the GIL cultures of patients with GVD was remarkable, in contrast to the non-detectable levels of the cultures from patients without GVD after the first year after HTx. Tilney and Hancock[14,15] also described the importance of IL-6 after the diagnosis of chronic rejection in rats. When more then 50% of the glomeruli were enlarged from mesangial proliferation, the glomeruli were stained positive for IL-2, IFN-γ, TNF-α, and IL-6. Even when most glomeruli were sclerotic, little cytokine activity was noted, except for IL-6. Russel et al.[16] detected early and sustained expression of IL-6 by immunohistology in a rat model of chronic rejection after HTx. This increase in IL-6 was not only detected in the infiltrate, but also in the serum of the rats.[14] However, in the serum and urine of human renal allografts recipients, no relation between IL-6 and chronic rejection was seen.[26] We suggest that the IL-6 produced by the GIL derived from patients with GVD will continue the process of intima thickening in the vessels of the donor heart. Because IL-6 promotes the proliferation of smooth muscle cells,[27] this may be a co-factor in the accelerated process of coronairsclerosis.

In summary, we presented evidence that GVD is an immune-mediated response to donor antigens and that the development of GVD is initiated by Th1 cytokines produced by GIL early after transplantation, whereas the IL-6 production of GIL maintains and accelerates GVD.

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Chapter 4.3

The frequency and avidity of committed cytotoxic T-lymphocytes (cCTL) for donor HLA class I and class II antigens and their relation with graft vascular disease

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Abstract

Background: Cellular immune processes may trigger the development of graft vascular disease (GVD). CD4 and CD8 cytotoxic T-lymphocytes that infiltrate the allograft could play a role in the development of GVD.

Methods: We studied the presence of *in vivo* primed or committed cytotoxic T-lymphocytes (cCTL) and their avidity for donor HLA class I and class II antigens in graft-infiltrating lymphocyte cultures propagated from endomyocardial biopsies derived from patients with and without signs of GVD. The fraction of cCTL with high avidity for HLA class I or class II antigens was estimated by the addition of anti-CD8 or anti-CD4 monoclonal antibodies (MoAb) to the cytotoxic phase of the limiting dilution analysis.

Results: In the first year after transplantation no difference in the frequency of donor-specific class I cCTL between patients with and without GVD was found. Addition of anti-CD8 mAb revealed that most cultures predominantly consisted of cCTL with low avidity for donor HLA class I antigens, irrespective of the development of GVD at one year after transplantation. However, in patients who did not develop GVD, the frequency of cCTL with donor HLA class II specificity was significantly higher than in patients who did develop GVD. The avidity for donor HLA class II antigens was comparable in both groups.

Conclusions: A high frequency of donor-specific cCTL for HLA class II antigens seems to be a protective factor against the development of GVD. These cCTL might be cytotoxic for cells involved in GVD development, e.g. activated endothelium and smooth muscle cells of donor origin.

Introduction

The long-term success of organ transplantation is limited by the development of chronic rejection. In the allograft, chronic rejection manifests itself as an occlusive disease of the donor vessels, which leads to ischemia and thereby to organ dysfunction. In human cardiac allografts, it is characterized by a process of accelerated coronary artery disease, which can be visualized by angiography or intravascular ultrasound. The vascular lesions develop in the donor vessels, while the recipient vessels remain free from lesions. Therefore an allogeneic immune process is probably involved in the pathogenesis of graft vascular disease (GVD). Cells obtained from affected coronary vessels are a likely source to study cellular immune processes in relation to GVD. Unfortunately, such an analysis is limited by the material available. However, GVD involves vessels of different sizes in the graft,[1] including the very small vessels that can be observed in endomyocardial biopsies (EMB).[2,3] After heart transplantation (HTx), EMB are regularly taken to diagnose acute rejection. Recently, we have demonstrated that during the first post-operative year graft-infiltrating lymphocytes (GIL) propagated from EMB derived from patients who developed GVD at one year after HTx produced significantly more T-helper 1 cytokines than the cultures from patients who remained free from GVD.[4] Not only the T-helper cells, but also cytotoxic T-lymphocytes (CTL) could play a role in the development of GVD. During acute rejection, donor-specific CTL propagated from EMB had mainly a high avidity for donor HLA class I and class II antigens, whereas CTL from non-rejection EMB had a low avidity for donor antigens.[5-7] The CTL with high avidity for donor HLA class I or class II antigens are resistant to *in vitro* inhibition with CD8 or CD4 monoclonal antibodies (MoAb), respectively, indicating that these cells do not need the CD8 or CD4 molecule to stabilize their antigen binding.[8,9] On the other hand, low-avidity CTL can easily be inhibited.
In the present study, we analyzed the cytotoxic capacity of GIL to donor HLA class I and class II antigens during the first post-operative year and their relation with GVD as diagnosed at one year after HTx. To study the relevance of $CD8^+$ and $CD4^+$ CTL during the development of GVD, we investigated the frequency of *in vivo* primed or committed CTL (cCTL) present within the graft and their avidity for donor HLA class I and class II antigens in the first year after HTx, thus before the diagnosis of GVD.

Patients and methods

Patients
We studied 89 cardiac allograft recipients transplanted consecutively between September 1987 and January 1991. Detection of acute rejection was performed by histological grading in EMB and defined as mononuclear cell infiltrates with myocyte damage. We refer to acute rejection when grade 3A or more is histologically diagnosed.[10] At one year after HTx, 18 patients had signs of GVD and 71

patients did not. GVD was visually assessed by coronary angiography taken at one year after HTx, and scored by one of us (AHMM B). GVD was defined as all vascular wall changes, including minimal wall irregularities.
All patients received preoperative blood transfusion, maintenance immunosuppression consisted of cyclosporin A (CsA) and low dose of steroids.

Culturing, phenotypic analysis and cell-mediated lympholysis of cultures
GIL were established from EMB in 96-well U-bottomed tissue culture plates (Costar, Cambridge, MA) in medium (RPMI-1640 Dutch Modification; Gibco, Paisly, UK) supplemented with 10% pooled heat-inactivated human serum, 4mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (=culture medium) in the presence of approximately 30 U/ml exogenous IL-2 (lectin-free lymphocult T-LF; Biotest AG, Dreieich, Germany) and $1x10^5$ irradiated (30 Gy) autologous PBMC (peripheral blood mononuclear cells) per well.[11] GIL-cultures propagated under these conditions contain *in vivo* primed or cCTL.[6,12] The plates were incubated in a humidified atmosphere at 37 °C in 5% CO_2.
When sufficient cell numbers was reached, the cultures were analyzed by three-colour flow cytometry on a FACScan (Becton and Dickinson, San Jose, CA) for the expression of cell surface markers. Screening was performed with the combination WT31 FITC (T-cell receptor-αß), CD4 PE and CD8 PERCP.
Subsequently, the cytotoxic capacity of GIL cultures was tested against donor cells or a panel of target cells sharing either HLA class I or class II antigens with the donor. Briefly, effector GIL were incubated with $2.5x10^3$ ^{51}Cr-labelled target cells at different effector/target ratios in 200 µl culture medium. After 4 hours of incubation supernatants were harvested and the release of ^{51}Cr-release was determined as described in the limiting dilution analysis (LDA). Cells not used for this test were stored at -140 °C.

Allogeneic target cells
T-cell blasts were obtained by culturing donor spleen cells for 7 days in culture medium supplemented with 1% phytohaemagglutinin (PHA)-M (Difco, Detroit, MI), after 3 days half of the medium was replaced by culture medium supplemented with 10% v/v lymphocult-T (Biotest). These blasts served as target cells to determine donor class I-directed cytotoxicity. The T-cell blasts can not be used as HLA class II targets.[7]
Epstein-Barr virus (EBV)-transformed B-cell lines (B-LCL) were obtained by infection of PBMC or spleen cells with the virus from the marmoset cell line B95-8 and addition of CsA as described by Moreau et al.[13] These cells were maintained in culture medium supplemented with 10% heat-inactivated bovine calf serum (BCS; Hyclone, Logan, Utah). The B-LCL served as target cells to measure HLA class I and II antigens. Because we determined especially the avidity for donor HLA class II antigens, we used B-LCL that did not share donor HLA class I, but shared only donor HLA class II antigens with the donor.[7]

Limiting Dilution Analysis
We obtained outgrowth of GIL in approximately 42% of the non-rejection EMB[14] and stored the cultures when enough cells were left after the standard cell-mediated lympholysis test. Only cultures showing donor-specific class I and/or class II cytotoxicity were analysed in the present study. We studied 19 GIL cultures propagated from EMB of 8 patients without GVD and 10 patients with signs of GVD in their one-years angiogram. The donors of these patients had several mismatches for HLA class I (HLA-A and -B) and/or HLA class II (HLA-DR) antigens (Tables 1 and 2). The selected cultures had to exhibit donor HLA class I-directed cytotoxicity in ^{51}Cr-release assays in order to be able to determine the percentage of cells with high avidity for donor HLA class I antigens, and donor HLA class II-directed cytotoxicity to determine the percentage of cells with high avidity for donor HLA class II antigens. The days after HTx, days in cultures, phenotypic composition and number of HLA mismatches (mm) between donor and acceptor were comparable between the patients with and without GVD (Tables 1 and 2).

The cultures were thawed in culture medium and irradiated (30 Gy) third-party B-LCL ($5x10^3$/well) were added as feeder. These B-LCL did not share HLA antigens with the donor and acceptor, to avoid *de novo* activation *in vitro* of precursor CTL that can mature to cCTL by restimulating with donor antigens. Thus, only the activity of *in vivo* activated cCTL were quantified in the LDA. When sufficient cell numbers were reached, limiting dilution cultures were set up in 96-well U-bottomed tissue culture plates (Costar).

Table 1 Characteristics of the graft-infiltrating lymphocyte cultures of which the fraction of cCTL and their avidity for donor HLA class I antigens were determined.

patient	pHTx (days)	culture (days)	WT31 %	CD3 %	CD4 %	CD8 %	CD16 %	γδ %	HLA-A mm	HLA-B mm	HLA-DR mm
					without GVD						
AN	163	60	97	97	40	53	0	0	1	2	2
BE	215	60	79	81	9	65	19	0	2	1	1
BN	216	29	88	89	5	76	10	1	2	2	2
HO	133	24	99	99	4	95	0	0	1	1	1
KU	196	23	74	95	15	77	4	20	1	2	2
					with GVD						
LI	212	37	99	99	49	50	0	0	1	2	2
SA	334	32	97	98	4	96	1	0	1	1	1
WI	141	30	90	90	29	58	2	0	1	1	1
WL	131	27	88	95	8	80	5	7	1	1	2
ZE	181	60	89	94	85	12	9	0	2	2	1

Graded number of responder GIL were titrated in 8 double dilutions starting from 5000 to 39 cells per well in 24 replicates with $5x10^4$ irradiated (30 Gy) autologous PBMC as feeder in 200 µl culture medium supplemented with 20 U recombinant IL-2 (Biotest). After 7 days of culture, the microcultures were split in two. Half of the split wells were tested in the absence of MoAb and the other half were tested in the presence of CD8 MoAb in case of determining the avidity for donor HLA class I antigens and CD4 MoAb for determining the avidity for donor HLA class II antigens. Each well was individually tested for cytolytic activity against $2.5x10^3$ ^{51}Cr-labelled target cells. T-cell blasts of donor origin were used as targets when the avidity for donor HLA class I antigens was tested. B-LCL were used as targets when the avidity for donor HLA class II antigens was tested.[7] These B-LCL lacked donor HLA class I molecules. After 4 hours of incubation at 37 °C in 5% CO_2 the supernatants were harvested (Skatron harvesting system: Skatron-AS, Lier, Norway) and the release of ^{51}Cr was determined in a γ-counter (Packard Instruments, Downers Grove, IL). Maximum and spontaneous release were determined in 4-fold and defined by incubation of target cells with culture medium in the presence and absence of Triton X-100 (5% v/v in TRIS buffer), respectively.
The percentage of specific lysis was calculated according to the formula:

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

The fraction of cCTL with high avidity for donor class I (CD8) or class II (CD4) HLA antigens was calculated using the formula:

$$\% \text{ cCTL with high avidity} = 100\% \times \frac{\text{cCTLf with CD8 or CD4 MoAb}}{\text{cCTLf without CD8 or CD4 MoAb}}$$

The microcultures were considered cytolytic when the percentage lysis exceeded 10%.
As a control for specificity, 5000 cells per well were cultured in quadruple and the capacity to lyse third-party PHA blasts or B-LCL (these targets cells did not share HLA antigens of the donor) and the K562 cell line was tested.

Table 2 Characteristics of the graft-infiltrating lymphocyte cultures of which the fraction of cCTL and their avidity for donor HLA class II antigens were determined.

patient	pHTx (days)	culture (days)	WT31 %	CD3 %	CD4 %	CD8 %	CD16 %	γδ %	HLA-A mm	HLA-B mm	HLA-DR mm
					without GVD						
BN	216	29	88	89	5	76	10	1	2	2	2
BR	251	40	99	98	74	26	0	1	2	2	2
BU	218	34	99	99	67	33	0	0	0	1	1
KU	196	23	74	95	15	77	4	56	1	2	2
MO	35	26	96	96	54	46	4	0	2	2	1
					with GVD						
BO	128	55	97	97	98	0	2	0	2	2	2
DA	213	36	99	99	64	38	0	0	2	1	2
FR	122	25	87	87	45	42	15	0	1	2	1
JA	225	29	100	100	13	87	0	0	1	2	2
LI	212	37	99	99	49	50	0	0	1	2	2
VE	21	25	94	94	42	58	3	0	2	2	1
WI	141	30	90	90	29	58	2	0	1	1	1
WL	131	27	88	95	8	80	5	7	1	1	2
ZE	224	79	98	91	10	91	0	0	2	2	1

CD8 inhibition
FK18 (a kind gift of Dr F. Koning, Department of Immunohematology and Bloodbank, University Hospital Leiden, The Netherlands), a mouse antibody of the IgG3 subclass, recognizes the gp32 chain of the human CD8 molecule.[15] A 1:500 dilution of ascitic fluid totally inhibited the cytotoxic capacity of CD8-dependent CTL clones, but did not affect target cell lysis by CD8-independent CTL clones. CD8 clones were not affected by the CD4 MoAb RIV6.[7] Before addition of the target cells to

the microcultures, FK18 was added to half of the split well cultures and were incubated for 30 minutes at 37 °C in a humidified atmosphere containing 5% CO_2.

CD4 inhibition

RIV6 (a kind gift of Dr M.F. Leerling, RIVM, Bilthoven, The Netherlands) is a mouse antibody of the IgG2a subclass directed against the human CD4 molecule. At a concentration of 1 µg/ml, RIV6 totally inhibited the cytotoxic capacity of CD4-dependent CTL clones, but did not affect target cell lysis by CD4-independent CTL clones. CD4 clones were not affected by FK18.[7] Before addition of the target cells to the microcultures, RIV6 was added to half of the split well cultures and were incubated for 30 minutes at 37 °C in a humidified atmosphere containing 5% CO_2.

Statistical analysis

Data concerning the cytotoxicity against donor HLA class I and class II antigens were analyzed with the Fischer's Exact Test (Tables 3 and 4). The CTL frequency (CTLf; expressed as the number of CTL per 10^6 cells) and the 95% confidence interval (CI) were calculated by the Jackknife procedure for maximum likelihood.[16] The calculated frequencies were accepted when the goodness-of-fit did not exceeded 12. The significance of differences between the patient groups was analyzed with the Mann-Whitney U-test.

Results

GIL with donor-specific HLA class I and class II CTL

We studied from 89 patients 531 GIL cultures propagated from acute rejection and non-rejection EMB, and analyzed per patient (Table 3) and per culture (Table 4) the cytotoxic reactivity for donor HLA class I and class II antigens. Moreover, we investigated whether this cytotoxicity was discriminating for GVD. There was no difference in non-donor-specific cytotoxicity, donor HLA class I or class II, or both donor HLA class I and II cytotoxicity of the GIL cultures between the patients with and without GVD, neither when the cytotoxic reactivity per patient was analyzed (Table 3A) nor when all cultures together were analyzed (Table 4A). The cytotoxic capacity during acute rejection episodes was not discriminating for GVD development (Tables 3B and 4B).

The frequency of cCTL for donor HLA class I and their avidity for class I antigens

We determined the frequency of cCTL and the fraction of high-avidity cCTL specific for donor class I antigens in 5 cultures from patients with GVD and 5 cultures from patients without GVD. Since we found previously that acute rejection correlated with an increased number of high-avidity CTL for donor HLA class I antigens,[5,6] we only analyzed cultures derived from non-rejection EMB.

The cCTLf reactive to target cells expressing donor HLA class I antigens was not different between the two patient groups (Table 5). When anti-CD8 MoAb were added during the CML phase also no difference in cCTLf was found (Table 5). Looking at the avidity of the cCTL, we demonstrated that most cultures during the

first year after HTx predominantly consisted of cCTL with low avidity for donor HLA class I antigens, irrespective of GVD development at one year after transplantation (Figure 1A).
The cCTL did not lyse third party PHA blasts or B-LCL and the K562 cell line. Therefore we conclude that the cytotoxic capacity was donor-specific.

Table 3 Donor-specific cytotoxic characteristics analyzed per patient of graft-infiltrating lymphocyte cultures from patients with and without graft vascular disease (GVD) during the first year after transplantation.

A. Non-rejection endomyocardial biopsies (EMB)

	without GVD	with GVD
no. of patients	71	18
no. of patients of whom none of the cultures were cytotoxic for donor antigen	4	0
donor HLA class I cytotoxicity	65/67*	18/18
donor HLA class II cytotoxicity	57/67	14/18
both donor HLA class I and II cytotoxicity	55/67	14/18

B. Acute rejection EMB

	without GVD	with GVD
no. of patients	37	10
no. of patients of whom none of the cultures were cytotoxic for donor antigen	5	1
donor HLA class I cytotoxicity	31/32*	9/9
donor HLA class II cytotoxicity	24/32	5/9
both donor HLA class I and II cytotoxicity	23/32	4/9

*number of patients with at least one culture with cytotoxicity

The cCTL frequency of donor HLA class II and their avidity for class II antigens

The cCTLf and the fraction of high-avidity cCTL for donor class II antigens were measured in 9 patients with GVD and 5 patients without GVD. Acute rejection was already found to be associated with high-avidity CTL for donor HLA class II antigens in a previous study.[7] Therefore we only determined GIL cultures derived from non-rejection EMB.
In patients who did not develop GVD at one year after transplantation the cCTLf with donor class II specificity was significantly higher than the frequency in the patients who had GVD (Table 6). Also, in the presence of anti-CD4 MoAb significant higher

frequencies were found in patients without GVD (Table 6). The percentage cells with high avidity in the cultures for donor HLA class II antigens was comparable between the patient groups (P=0.19) (Figure 1B).
Remarkable was the higher frequency of cCTL directed against donor HLA class II antigens compared with those cCTL directed against donor HLA class I antigens in patients who remained free from GVD (P=0.10) (Tables 5 and 6).

Table 4 Donor-specific cytotoxicity analyzed per graft-infiltrating lymphocyte culture from patients with and without graft vascular disease (GVD) during the first year after transplantation.

A. Non-rejection endomyocardial biopsies (EMB)

	without GVD	with GVD
no. of cultures tested	365	94
no. of cultures which were not cytotoxic	88	18
donor HLA class I cytotoxicity	250/277	70/76
donor HLA class II cytotoxicity	158/277	43/76
both donor HLA class I and II cytotoxicity	128/277	35/76

B. Acute rejection EMB

	without GVD	with GVD
no. of cultures tested	57	15
no. of cultures which were not cytotoxic	5	3
donor HLA class I cytotoxicity	46/52	11/12
donor HLA class II cytotoxicity	34/52	5/12
both donor HLA class I and II cytotoxicity	30/52	4/12

Discussion

Chronic rejection is a frequently encountered complication after organ transplantation. The pathogenesis of GVD is still unknown. However, as the vascular lesions develop in donor vessels and remain absent in recipient vessels, allogeneic immune processes may be involved in the pathogenesis of GVD. In the search for immunological parameters, we found no relation between GVD and the number of acute rejection periods, preoperative reactive antibodies against a panel of lymphocytes, number of HLA mismatches, *in vitro* growth or phenotypic composition of GIL derived from EMB.[14,17] We found, however, that certain characteristics of

donor-specific T-helper cells are associated with the occurrence of GVD. Both IL-2 mRNA expression in EMB[18] and production of high levels of T-helper 1 cytokines (IL-2 and IFN-γ) by GIL derived from EMB[4] were especially found in patients with GVD. In the present report we investigated the possible link between GVD and the frequency or nature of CTL propagated from GIL.

Table 5 Frequencies of donor-specific cCTL with their percentage of CD8 inhibition in graft-infiltrating lymphocyte cultures from patients with or without graft vascular disease (GVD).

patient	without anti-CD8		with anti-CD8		inhibition
	cCTLf	95% CI	cCTLf	95% CI	(%)
		without GVD			
AN	604	458-750	218	147-288	64
BE	153	72-234	0,00	0-0	100
BN	1671	1239-2104	721	540-901	57
HO	200	130-270	58	24-92	71
KU	130	78-181	88	46-130	32
		with GVD			
LI	8267	6338-10197	3277	2579-3976	60
SA	630	437-823	600	418-783	5
WI	222	156-287	80	40-120	64
WL	1207	798-1616	382	280-484	68
ZE	28	5-50	0,00	0-0	100

First, we studied whether the presence of donor-directed CTL within the transplanted heart is associated with GVD. We found that the frequency of the GIL cultures lytic to donor HLA class I and class II antigens does not discriminate patients with GVD from patients who remain free from GVD in the first year after transplantation.
Secondly, we analyzed 19 cultures from 10 patients with GVD and 8 patients without signs of GVD in more detail. We found no differences in phenotype of the GIL cultures between patients with and without GVD. This confirms our previous study, which also showed that the phenotypic composition of the cultures does not correlate with GVD.[14] The EMB-derived cultures consisted of a mixture of CD8 and/or CD4-resistant and -susceptible donor-reactive CTL. During the first year after HTx, the frequency of donor-specific HLA class I cCTL did not differentiate the patients with

GVD from those without GVD. The same was true when the cytotoxicity was studied in the presence of CD8 MoAb. The cultures contained predominantly cCTL with low avidity for donor HLA class I antigens, irrespective of the development of GVD at one year after HTx. During the first post-operative year, the frequency of cCTL to HLA class II donor antigens was found to be significantly higher in patients who remained free from GVD than in patients with angiographic signs of GVD at one year after transplantation. The fraction cCTL with high avidity for donor HLA class II antigens in the GIL cultures was comparable between these patient groups.

Table 6 Frequencies of donor-specific cCTL with their percentage of CD4 inhibition in graft-infiltrating lymphocyte cultures from patients with or without graft vascular disease (GVD).

patient	without anti-CD4		with anti-CD4		inhibition
	cCTLf	95% CI	cCTLf	95% CI	(%)
			without GVD		
BN	335[a]	243-427	420[b]	318-523	0
BR	1519	1138-1901	649	436-862	43
BU	5266	3775-6756	731	527-934	86
KU	3928	3006-4849	5375	4149-6602	0
MO	818	563-1073	229	157-301	70
			with GVD		
BO	174	115-233	56	25-87	68
DA	783	590-976	36	7-65	95
FR	208	138-279	134	80-187	36
JA	627	436-818	175	110-241	72
LI	138	84-192	30	4-56	78
VE	818	622-1014	422	319-524	48
WI	95	51-139	0	0-0	100
WL	941	709-1174	620	471-769	34
ZE	320	218-423	19	0-39	94

[a]cCTLf obtained in the absence of CD4 of patients without GVD compared to those of with GVD (P=0.02)
[b]cCTLf obtained in the presence of CD4 of patients without GVD compared to those of with GVD (P=0.01)

These data suggest that a high frequency of donor HLA class II-directed cCTL protects for GVD. As a possible explanation for this phenomenon, we suggest the following model. Shortly after transplantation, MHC class II antigens are predominantly expressed on the vascular wall of donor origin[19] due to tissue damage as the result of ischemia and reperfusion.[20,21] Macrophages infiltrate the graft (GIL) and release cytokines to their microenvironment. These cytokines cause an upregulation on donor cells of donor HLA class I and class II antigens and expression of lymphocyte binding ligands (intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), etc.) in the graft. The permeability of the microvessels increases and donor-specific activated mononuclear cells (macrophages and lymphocytes) enter the graft. Subsequently, in patients who remain free from GVD, allo-activated cCTL specific for donor HLA class II antigens accumulate at sites with activated donor cells. Thereafter donor cells with HLA class II antigens on their surface, which are responsible for the development of GVD (endothelial cells and smooth muscle cells), are killed, thereby preventing the generation of GVD. At the same time the frequency of cCTL specific for donor HLA class I antigens is still too low to be cytotoxic. Previously, our research group demonstrated that cCTL from GIL are able to lyse donor endothelial cells.[22]

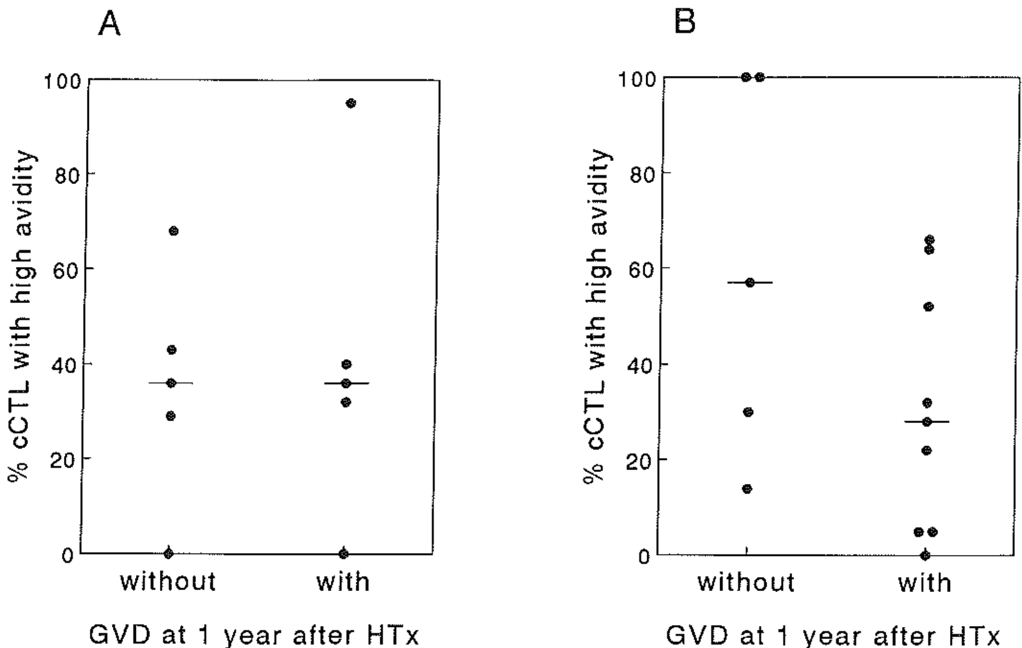

Figure 1 The fraction of graft-infiltrating lymphocyte cultures propagated from endomyocardial biopsies taken during the first pre-operative year from patients with and without graft vascular disease (GVD) at one year after transplantation having a high avidity for donor HLA class I (A) and class II (B) antigens.

We have also reported a high production of T-helper 1 cytokines by GIL derived from patients with GVD in the first year after HTx compared to those from patients without GVD.[4] This consequently results in an up-regulation of HLA class I and class II antigens on vascular endothelial cells of patients with GVD.[23] In case of GVD, the frequency of both the donor-directed HLA class I and class II cCTL is apparently too low to lyse the activated vascular wall cells and the characteristic process of GVD development continues: the activated endothelial cells and mononuclear cells secrete growth factors, which promote proliferation of smooth muscle cells, ultimately leading to intima thickening.
Dong et al.[24] demonstrated that indeed endothelial cell damage was less evident in the vessels with greater intimal disease severity, probably by a decreased number of HLA class II specific CTL. In future, our proposed model for GVD has to be confirmed by an investigation of growth factor production by vascular cells in the presence of CTL from patients with and without GVD.
In conclusion, cCTL that infiltrate the human cardiac allograft could play a role in the development of GVD. Especially the high frequency of donor-specific cCTL for HLA class II antigens seems to be a protective factor in the development of GVD.

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Chapter 5

Peripheral blood monitoring of acute rejection and graft vascular disease

Chapter 5.1

Clinical relevance of peripheral monitoring of direct and indirect antigen-presentation pathways after heart transplantation

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NM van Besouw, LMB Vaessen, AHMM Balk, B Mochtar, FHJ Claas, W Weimar. **CsA therapy affects the direct and indirect antigen-presentation pathway in cardiac allograft recipients.** *Transplant Proc 1996; 28 (6): 3135-3136.*

NM van Besouw, CR Daane, LMB Vaessen, T van Gelder, B Mochtar, AHMM Balk, W Weimar. **Nonspecific immune reactivity of peripheral blood mononuclear cells is related to graft vascular disease.** *Transplant Proc 1997; 29: 2544-2545.*

Abstract

Background: Monitoring for the responses to alloantigens presented either by the direct or the indirect presentation pathway have been reported to be of clinical value after kidney transplantation. Amongst others the level of these responses may be dependent on the immunosuppressive treatment.
Methods: We studied these pathways in peripheral blood mononuclear cells (PBMC) of cardiac transplant patients in an attempt to find a correlation between these tests and the clinical status of the patients.
Results: Both before and after transplantation, comparable proportions of PBMC samples reacted in MLC (mixed lymphocyte cultures) to nondepleted donor spleen cells (direct route), but never to donor cells depleted for antigen-presenting cells (indirect route). In contrast, the latter route could easily be activated by a nominal antigen (tetanus toxoid (TET)) and persisted after transplantation. After transplantation the proportion of PBMC samples responding to TET was significantly suppressed, irrespective of the occurrence of rejection. Nevertheless, analyzing the level of PBMC reactivity, both the immune responses via the direct and indirect presentation route increased when cyclosporine A (CsA) levels inadvertently decreased to inadequate concentrations and acute rejection was diagnosed. During immunological quiescence high and low responders could be detected to both the direct and indirect pathway, while graft vascular disease (GVD) was only positively correlated with a higher PBMC reactivity to indirect presented TET antigens.
Conclusion: The level of PBMC reactivity via both the direct and indirect presentation pathway is correlated with acute rejection early after transplantation, while only the indirect pathway was associated with GVD. These tests might be used as a tool for selecting patients in which tapering of immunosuppresion can safely be performed.

Introduction

Alloantigens can only stimulate T-cells via antigen-presenting cells (APC). T-cells require two signals of the APC to become activated. An antigen specific signal is provided by the antigen peptide in context with major histocompatibility complex (MHC) molecules present on the cell surface of the APC and interacting with the T-cell receptor of the corresponding T-cell. A nonspecific second signal is given by interaction between adhesion and costimulatory molecules on the APC and on the responding T-cell. In order of potency dendritic cells, macrophages, B-cells, and in some circumstances activated endothelial cells, T-cells, keratinocytes, fibroblasts, and eosinophils do have antigen-presenting capacity.[1-5] After transplantation, both donor APC (dAPC) and recipient APC (rAPC) are available to cause an immune response. dAPC differ from rAPC in MHC molecules expressed on their surface (donor or recipient MHC) and in proteins that were dissociated from foreign peptides derived from pathogens in the donor. T-helper cells of the transplant recipient may respond to dAPC via the direct presentation pathway or to rAPC via the indirect presentation pathway (reviewed by Sayegh).[6] In case of the direct pathway, T-cells recognize allogeneic MHC molecules on the surface of dAPC. For the indirect pathway donor MHC molecules are internalized by rAPC, subsequently processed into peptides which are bound by recipient's MHC class I and class II molecules, and finally presented at the cell surface of the rAPC to self-MHC-restricted allopeptide specific responder cells. This last pathway is identical to the presentation of nominal antigens, e.g. tetanus toxoid (TET).

In vivo, the direct alloantigen presentation pathway is thought to be responsible for acute rejection and the indirect pathway for chronic rejection. As a consequence of the gradually replacement of dAPC by rAPC,[7] in time less dAPC are able to stimulate the recipient T-cells. However, the already activated T-cells become memory T-cells and may be susceptible to donor cells (endothelial cells) of the graft and do not need professional APC (dendritic cells, macrophages, activated B-cells). Others suggest that human endothelial cells, that constitutively express MHC class II antigens, have even the ability to provide all the signals necessary for direct stimulation of resting allogeneic T-cells.[4,5] Thus both pathways may remain present in the long term after transplantation.

In vitro, both presentation pathways were found to be able to induce an alloresponse by peripheral blood mononuclear cells (PBMC) of healthy volunteers.[8] In renal transplant recipients with stable graft function, the direct pathway was not different from that of healthy donors, whereas these patients showed no response to alloantigens presented via the indirect pathway. When the latter reactivity was detected, a correlation with acute or chronic rejection was reported.[9,10] In addition, it

has been suggested that the indirect pathway is more sensitive to immunosuppression than the direct route of alloantigen presentation.[10-12]

The present overview is mainly based on the manuscripts we published on presentation pathways,[13-16] and some new results are added. We monitored the presence of both antigen presentation pathways in PBMC of human cardiac transplant recipients, by measuring the proliferative response of their cells to alloantigens in mixed lymphocyte cultures (MLC) and to TET. PBMC samples were collected longitudinally from the time of heart transplantation (HTx) to 2 years thereafter. In order to test whether the magnitude of the response of the PBMC to the different presentation pathways is dependent on the immunosuppressive load,[8-11] we also measured these responses before, during and after a moment that *in vivo* CsA (cyclosporine A) inadvertently decreased to inadequate plasma levels resulting in acute rejection. In addition, we wondered whether we could demonstrate differential PBMC reactivity to directly and indirectly presented antigens between patients with and without GVD (graft vascular disease). Finally, we determined the PBMC reactivity to the presentation routes in a period of immunological quiescence, in attempt to find a correlation with the immunological status of the patients and possibly to obtain a relevant tool to monitor the effect of tapering immunosuppression on the immune response.

Materials and methods

Patients

All patients received one preoperative blood transfusion. Standard immunosuppressive therapy, consisting of CsA and low dose prednisone, was given as maintenance immunosuppression.

Acute rejection was diagnosed histologically in endomyocardial biopsies and defined as cellular infiltrate with myocyte necrosis (ISHLT grade 3 or more)[17] upon which anti-rejection therapy was instituted.

One year after transplantation, GVD was visually assessed on coronary angiograms and defined as all vascular wall changes including minimal wall irregularities.[18]

PBMC sampling

PBMC samples were isolated from heparinized blood by density gradient centrifugation using Ficoll-Isopaque (δ=1.077; Pharmacia Biotech AB, Uppsala, Sweden). The PBMC were collected from the interphase, washed twice with Hanks' Balanced Salt Solution (HBSS, Gibco BRL, Scotland, UK) and frozen at -140 °C until use.

First, we determined the proportion of PBMC samples (n=68) from cardiac transplant recipients responding via the direct and indirect pathway before and after HTx.

We selected 14 PBMC samples from 3 transplant recipients, who experienced one or more periods of acute rejection during the first 4 months after HTx at a moment that CsA inadvertently decreased to inadequate concentrations. In these samples we measured quantitatively the proliferative response to both presentation pathways.

In addition, we measured the PBMC response to the presentation pathways of 5 patients with GVD

and 5 patients without GVD in the period 3 to 6 months, 1 year and 3 years after HTx. No histologic signs of acute rejection were seen in their endomyocardial biopsies taken at the same time of the PBMC samples.

Finally, we determined the PBMC reactivity to the presentation routes from 5 patients at the day before HTx and in a period of immunological quiescence (PBMC samples (n=28) taken from approximately 4 months till 2 years after HTx when no acute rejection occurred).

Donor cell sampling

Spleen cells were obtained by mechanical dissociation of small pieces of donor spleen through a sieve of stainless steel in RPMI 1640-DM supplemented with 4 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (all from Gibco, Paisley, Scotland), and 10% pooled heat inactivated human AB serum (i.e. culture medium). DNase (10 µg/ml, Boehringer Mannheim GmbH, Germany) was added to prevent aggregation of cells. Subsequently the cell suspension was filtrated through a 70 µm cell strainer (Falcon, Franklin Lakes, NJ) and washed. Thereafter the cells were centrifuged over a Ficoll-Paque density gradient and stored at -140 °C.

Proliferation tests

To test the response to alloantigens presented via the direct or the indirect presentation route, we stimulated PBMC from the transplant recipient with spleen cells derived from the heart donor (direct route) or with donor spleen cells depleted for dAPC (indirect route). The response to TET (RIVM, Bilthoven, the Netherlands) served as positive control for the indirect presentation pathway.

100 µl of a PBMC suspension of $5x10^5$ cells/ml in culture medium (RPMI 1640-DM supplemented with 4 mM L-glutamine, 100 IU/ml penicillin and 10% heat inactivated human AB serum) was added in triplicate wells in a round-bottomed 96-well plate (Costar, Cambridge, MA) to 100 µl (a) $5x10^4$ irradiated (60 Gy) spleen cells derived from the donor; (b) $5x10^4$ irradiated (60 Gy) dAPC depleted spleen cells; (c) TET at 7.5 lf/ml final concentration; (d) phytohemagglutinin M (PHA, 1:100 final dilution; Difco Laboratories, Detroit, MI) to control the responsiveness of the cells; and (e) culture medium. To measure differences between patients with and without GVD, we stimulated the PBMC with Epstein Barr Virus transformed B-cell lines of donor origin (100 µl $5x10^3$ irradiated (30 Gy) donor B-cells) to determine the response of PBMC via the direct presentation pathway. After 6 days of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, cell proliferation was measured by incorporation of ^{3}H-thymidine (0.5 µCi/well) added during the last 8 hours of culture. The mean counts per minute (cpm) were determined and expressed as the stimulation index (SI). The SI is the ratio of the cpm obtained in the presence of antigen to the cpm obtained in the absence of antigen. Cell proliferation was considered significant when the SI was higher than 2.

APC-depletion

To remove all dAPC, 10^7 donor spleen cells (in 3.5 ml) were incubated with 125 µl of anti-CD14 (monocytes/macrophages; MY4, Coulter Immunology, Hialeah, FL), 75 µl of anti-CD19 (B-cells; Leu-12, Becton Dickinson, San Jose, CA) and 75 µl of anti-HLA-DR (dendritic cells; Becton Dickinson) in HBSS and 1% pooled heat inactivated human AB serum. After an incubation period of gentle shaking for 30 minutes at 4 °C, the cells were washed in HBSS. Thereafter the cells were incubated with 210 µl washed Dynabeads (M-450 sheep anti-mouse IgG, Dynal, Oslo, Norway) for 30 minutes of gentle shaking at 4 °C. Subsequently, the dAPC were retained by a magnet and the nonadherent cells were transported to another tube. This depletion procedure was repeated twice before the nonadherent cells were processed further. The APC-depleted cells were washed with HBSS and were counted with trypan blue to determine the vitality before testing.

HTL-frequency

The frequency of IL-2 producing T-helper lymphocytes (HTL) was determined using the limiting dilution

analysis assay (slightly modified) of Schanz et al.[19] In brief, 24 replicates of graded number PBMC responder cells were titrated in a four-step double dilutions starting from $5x10^4$ to 6250 cell/well and stimulated with $5x10^4$ irradiated (30 Gy) spleen cells depleted or not depleted for APC. After 3 days of culture, 100 µl of the culture supernatant were harvested to test for IL-2 in a bioassay using the IL-2 dependent CTLL-2 cell line as indicator system.

CsA levels

CsA trough levels were measured in plasma using a RIA with ^{125}I-labeled CsA and CsA-specific monoclonal antibodies (Cyclo-Trac SP; Incstar, Stillwater MN, USA). CsA plasma levels were maintained at therapeutic concentrations of 100-150 ng/ml after transplantation. Levels below 100 ng/ml were considered inadequate.

Results

Efficiency of APC-depletion

We have checked for the efficiency of APC-depletion by FACS-analysis and by performing two-way MLC with two depleted stimulator populations. After depletion 0% CD14 positive, <1% CD19 positive and <1% HLA-DR positive cells were detected by analysis on FACS and in none of the cases a response was found in the two-way MLC (stimulation index (SI) ≤2).

Discrepancy between stimulation of PBMC with APC depleted cells and TET

The results of capability of 68 PBMC samples to react via the direct or the indirect pathway in MLC are shown in Table 1. Most PBMC samples taken before HTx reacted to both nondepleted donor spleen cells (88%) and TET (75%), but never to spleen cells depleted for dAPC. Similarly after HTx, 75% (45/60) of the PBMC samples did respond to spleen cells not depleted for dAPC, but not to the depleted population, independent of time after transplantation or occurrence of acute rejection.

In contrast, the response to TET changed both after HTx compared with before HTx, and during the posttransplantation follow-up period. During the first year after HTx only 13% (6/47) of the PBMC samples reacted to TET, which is significantly less than before HTx (P<0.001). During rejection and nonrejection episodes, no significant difference in the TET response was observed (P=0.33). Also, no difference between the first and the second year after HTx was found with regard to the TET response of samples taken during rejection. In contrast, in periods without rejection, a trend was observed in time after HTx, i.e. the TET response was more frequently present during the second year (27%) compared with the first year (9%) after transplantation (P=0.15).

Frequencies of IL-2 producing helper T-lymphocytes (HTL)

We tested the frequency of HTL of 4 pre HTx samples, because the highest responses were expected at that point in time.[8] All PBMC reacted clearly to their nondepleted donor cell population (57, 33, 77, and 56 HTL/10^6 cells), while responses after APC depletion were nondetectable or very low (6, 0, 17, and 0 HTL/10^6 cells, respectively).

Table 1 Proliferative responses of PBMC samples pre and post heart transplantation (HTx) to directly and indirectly presented donor antigens and indirectly presented tetanus toxoid (TET) antigens.[a]

	pre-HTx	First year post-HTx		Second year post-HTx	
Stimulator[b]		No rejection	Rejection	No rejection	Rejection
directly presented donor antigens	7/8	25/34	9/13	9/11	2/2
indirectly presented donor antigens	0/8[c]	0/34[d]	0/13[d]	0/11[d]	0/2
indirectly presented TET antigens	6/8	3/34[e,f]	3/13[e,f]	3/11[e,f,g]	1/2[g]

[a]Data are presented as number of samples responding (stimulation index >2) per number of samples tested.

[b]The mean response of spleen cells not depleted for APC: 7,320 cpm (range, 170-44,719). Mean cpm without stimulus: 130 (range, 26-497). Mean response of TET: 1,928 cpm (range, 142-6,870).

[c,d]Response to nondepleted versus depleted spleen cells before ($P=0.001$)[c] and after HTx ($P<0.001$)[d] (two sided p-value: Fisher's exact test with Yates' correction).

[e]Response to nondepleted spleen cells versus TET during the first year after HTx in periods without ($P<0.001$) and with ($P=0.05$) rejection, and during the second year post HTx in periods without rejection ($P=0.03$).

[f]TET response before HTx versus TET response in the first year after HTx in nonrejection episodes ($P<0.001$) or in rejection episodes ($P=0.03$).

[g]TET response in the first year after HTx versus the second year after HTx during nonrejection ($P=0.15$) and rejection ($P=0.48$) episodes.

Acute rejection due to inadequate CsA levels in relation to antigen presentation pathways

Patient 1 (Figure 1A) experienced a rejection episode on day 75 after HTx. On this day a decrease in the level of CsA (86 ng/ml CsA) was found compared with the sample taken at day 40 (135 ng/ml CsA). At the same time the response to donor antigens mediated by the direct presentation pathway, increased from SI 1.4 (day 40) to 13 (day 75). The response to the indirect presented TET antigens was

undetectable (SI<2), this in contrast to the situation before HTx (SI=11). After anti-rejection therapy with steroids and increasing the dose of CsA, the response via the direct presentation route slowly decreased to a SI of 4.3 at day 97 when CsA reached the therapeutic level of 155 ng/ml.

Patient 2 (Figure 1B) had two successive biopsies with histological signs of acute rejection, on day 44 and day 51 after HTx. At both time points the CsA plasma levels, 43 and 88 ng/ml CsA, respectively, proved to be inadequate low with respect to the level at day 37 (175 ng/ml CsA). After treatment with steroids and adjusting the CsA

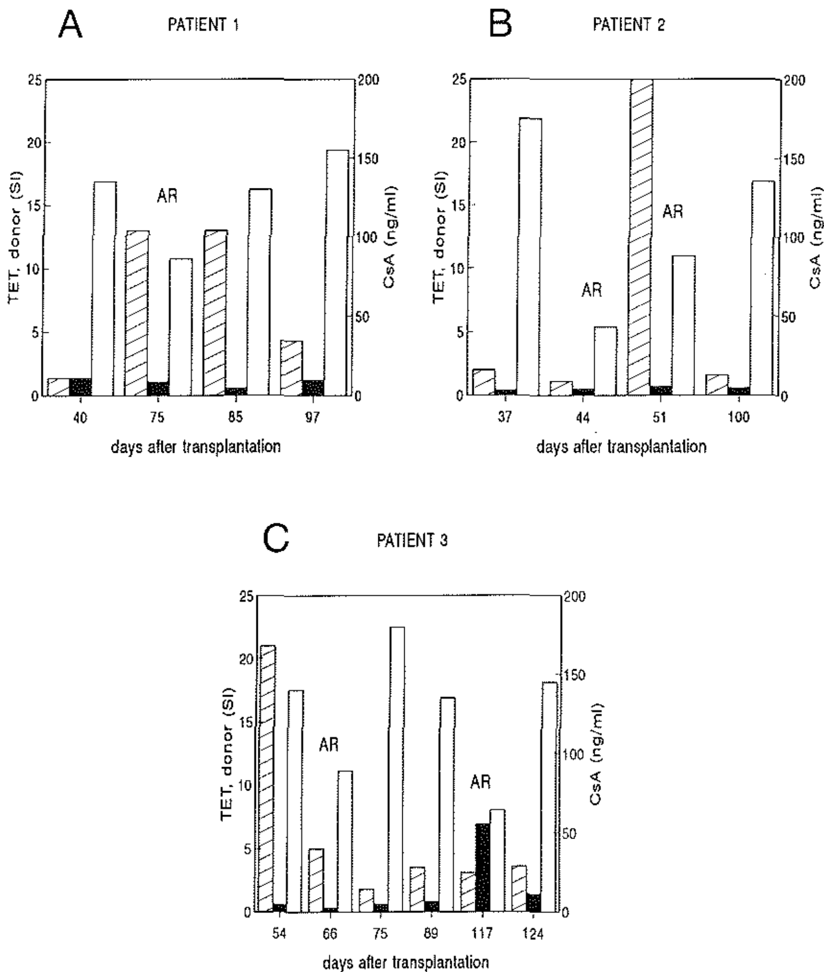

Figure 1 Relation with acute rejection (AR) and CsA levels in plasma (□), and with the proliferative capacity of PBMC to directly presented donor antigens (▨) and indirectly presented antigens (TET) (■).

dose on day 44, only a small increase in the CsA plasma level was detected on day 51 (88 ng/ml CsA), while the PBMC strongly responded to directly (SI=39) but not to indirectly (SI=0.7) presented TET antigens. A second anti-rejection therapy with rabbit anti-thymocyte globulin and a higher dose of CsA proved to be necessary. Thereafter, at day 100, the CsA concentrations in plasma reached normal therapeutic levels (135 ng/ml CsA), the response via the direct pathway became undetectable (SI=1.6) and no histological signs of rejection were found anymore.
Patient 3 (Figure 1C) experienced the first rejection period 66 days after HTx, again during a drop in CsA concentration in plasma (89 ng/ml CsA), while 12 days before a high reactivity of PBMC to directly presented donor antigens had been found (SI=21) and the indirect pathway was undetectable (SI=0.6). After anti-rejection treatment with steroids and increasing the CsA dose to reach a plasma level of 180 ng/ml CsA at day 75, the response via the direct pathway decreased to a SI of 1.8. On day 117 after transplantation a second rejection episode was observed, accompanied by another fall in the CsA level in plasma (64 ng/ml CsA) in combination with an increment of the response via the indirectly presented TET antigens (SI=6.9). In contrast to the first rejection episode, the response via the direct pathway remained low (SI=3.1). After anti-rejection therapy with steroids and an increased dose of CsA, the CsA levels normalized to therapeutic concentrations (145 ng/ml CsA) at day 124 and a reduction of the response of PBMC to the indirect pathway was found (SI=1.4).

Graft vascular disease and antigen-presentation routes

All PBMC samples of both patients with and without GVD reacted via the direct presentation pathway. However, not all PBMC samples reacted in a significant way via the indirect presentation route: 3 to 6 months after HTx, 3/5 patients with GVD and 1/5 patient without GVD; 1 year after HTx, 2/5 with GVD and 2/5 without GVD, and at 3 years after HTx 5/5 with GVD and 2/5 without GVD reacted to TET.
At 3 to 6 months, 1 year and 3 years after HTx, we neither found differences in the magnitude of the PBMC response to proliferate via direct presented donor antigens (Table 2A) between patients with and without GVD. In contrast, we found a relation with GVD and the indirect presentation pathway. Before (3 to 6 months after HTx) and after (3 years after HTx) the assessment of GVD at one year after HTx, the PBMC reactivity to TET was higher in patients with GVD compared to those who remained free from GVD (Table 2B).

Antigen-presentation pathways during immunological quiescence

During the second year after HTx, the PBMC response of patient 1 to both the direct presented donor antigens and indirect presented TET antigens increased compared to the PBMC reactivity before HTx (day 0) (Figure 2A). The PBMC reactivity of

patient 2 was only vigorous to the direct pathway (Figure 2B). The PBMC response of patient 3, 4 and 5 (Figure 2C, 2D, 2E) remained stable or even decreased to both pathways compared to the response before HTx.

Table 2 Proliferative responses of PBMC samples from patients with (n=5) and without (n=5) graft vascular disease (GVD) to directly presented donor antigens (A) and to indirectly presented tetanus toxoid (TET) antigens (B).

A

	3-6 months post-HTx	1 year post-HTx	3 years post-HTx
GVD-	82 (18-128)*	172 (27-559)	149 (60-1510)
GVD+	36 (7-73)	141 (7-670)	58 (8-352)

B

	3-6 months post-HTx	1 year post-HTx	3 years post-HTx
GVD-	0.7 (0.4-2.8)*	1.3 (0.6-5.2)	1.6 (0.7-9.4)
GVD+	6.2 (1.3-39)[1]	1.0 (0.7-14)	24 (2.5-143)[2]

*Data are presented as the median SI with the range in parentheses.
[1,2]TET response of patients with GVD compared to those without GVD (P=0.03[1], P=0.06[2]; two-sided P-value: Mann-Whitney U test)

Discussion

To investigate the clinical relevance of the allogeneic activation pathways after HTx, PBMC were stimulated with donor spleen cells not depleted for dAPC (direct pathway) or depleted for dAPC (indirect pathway) in MLC or were stimulated with TET antigens (indirect pathway). Most PBMC samples taken before HTx reacted to both nondepleted donor spleen cells and TET, but never to spleen cells depleted for dAPC. Similarly after HTx, only responses via the indirect presentation pathway to TET antigens were found and not to dAPC depleted spleen cells. In contrast to Shearer's group,[8-11] who found comparable positive responses both to indirectly presented nominal antigens and indirectly presented alloantigens in PBMC from kidney transplant recipients and healthy controls, we did not find any indirect response to alloantigens. Our results were confirmed by an unpublished study of PBMC from healthy persons, which did not react to an APC depleted stimulator population in MLC, while positive responses were found after stimulation with TET

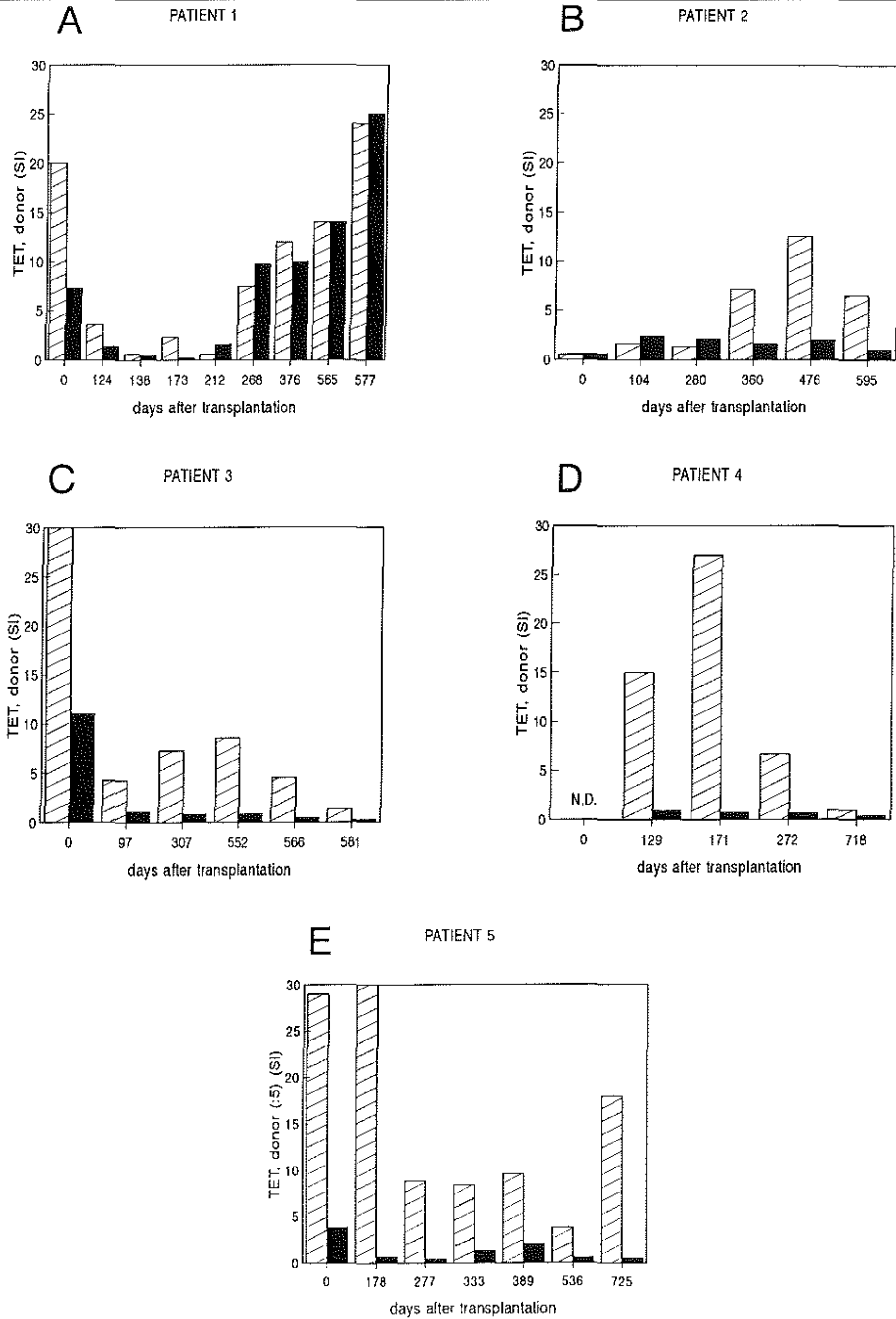

Figure 2 PBMC reactivity to directly presented donor antigens (▨) and to indirectly presented tetanus toxoid (TET) antigens (■) in an immunological quiescence period after heart transplantation (HTx).

(F.H.J. Claas, Immunohematology and Blood Bank, Leiden, personal communication, 1995). Although, the present study was different from that of Shearer's group with respect to the organ transplanted and the immunosuppressive regimen, we think that the different results are due to the different methods used for APC depletion. Depletion of APC by plastic and nylon wool adherence[8-11] may be incomplete, resulting in responses to suboptimal concentrations of dAPC, while the response was ascribed to alloantigens indirectly presented by rAPC. Dendritic cells are known as very potent APC, inducing MLC responses at low stimulator to responder ratios (1 dendritic cell to 1000 T-cells).[20] Accordingly, a slight contamination of the stimulator population with dendritic cells can already induce T-cell proliferation in a MLC. The reported correlation of dAPC depleted MLC responses with clinical events may rather be associated with the presence of a few remaining dAPC in the tests than with the measurement of the indirect presentation route. From the present study, it is clear that no responses to alloantigens in MLC can occur in the absence of dAPC.

An explanation for the positive responses of PBMC to the nondepleted cells and the negative responses of PBMC to the dAPC depleted population, however, remains that the direct presentation pathway is responsible for the strong proliferative responses seen *in vitro* by a high frequency of T-cell precursors responding to allogeneic MHC molecules.[21] The MLC used may not be sensitive enough for a detectable response to alloantigens presented via the indirect pathway to antigen specific T-cells. This was also reported by Watschinger et al.,[22] who indicated that T-cells primed *in vivo* by a vascularized cardiac allograft are able to proliferate *in vitro* to specific peptides but only when presented by APC, which were preincubated with the allopeptide. Therefore, we used a limiting dilution analysis assay to determine the HTL frequencies. This assay is thought to be more sensitive than MLC to measure responses to directly and indirectly presented antigens. We tested pre HTx samples, because the highest responses were expected at that point in time.[8] All PBMC clearly reacted to their nondepleted donor cell population, while responses after APC depletion were nondetectable or very low. However, interference of backward stimulation (i.e. IL-2 production of stimulator spleen cells in response to PBMC), as described by Schanz et al.[19] could have played a role in case of low HTL responses. To avoid backward stimulation, in general B-cell lines are used as stimulator cells in the HTL assay. However, these cell lines are not relevant for monitoring the indirect alloantigen presentation pathway, since they are excellent direct stimulators. Accordingly, as in the case with the MLC, the HTL assay with the APC-depleted cells as stimulator can not reliably be used to measure responses of PBMC to indirectly presented alloantigens.

Before transplantation comparable proportions of PBMC samples reacted via the

direct and the indirect route of presentation. After transplantation qualitative responses via the direct pathway (75%) were more frequently found than via the indirect pathway (13%), suggesting that CsA mainly affects the indirect route. In order to test whether the magnitude of the response of the PBMC to the different presentation pathways is dependent on the immunosuppressive load,[8-11] we measured these responses in a quantitative way before, during and after a moment that *in vivo* CsA inadvertently decreased to inadequate plasma levels resulting in acute rejection. During only one of these rejection episodes the indirect antigen presentation pathway was functional, while during the other 3 rejection periods only the direct pathway was found to be intact. This finding is consistent with data described by Muluk et al.,[10] who showed that the indirect presentation pathway is more susceptible to CsA therapy than the direct pathway. In agreement with these results, Clerici et al.[11] demonstrated that *in vitro* exposure of CsA to stimulated PBMC suppressed the indirect presentation pathway more strongly than the direct presentation pathway. Also Gallon et al.[12] confirmed that CsA inhibits proliferation of *in vivo*-primed T-cells to indirectly presented MHC peptides. While others suggested that only the indirect presentation pathway correlates with acute rejection, independently of the direct pathway,[10,11] we conclude from our data that acute rejection after clinical cardiac transplantation in the presence of inadequate CsA levels may be accompanied by an increase of the responses of PBMC both via directly and indirectly presented donor antigens. Therefore, we assume that after clinical heart transplantation CsA may prevent rejection by blocking both the direct and indirect presentation route.

We found a relation with GVD and the indirect, but not the direct presentation pathway. Before (3 to 6 months after HTx) and after (3 years after HTx) the assessment of GVD at one year after HTx, the PBMC reactivity to TET was higher in patients with GVD compared to those who remained free from GVD. Our data are in conflict with those from Reinsmoen et al.,[23] who found a relation between MLC donor-responsiveness (direct presentation) in the peripheral blood and chronic rejection in kidney, lung and heart transplant recipients. Others found a relation between the presence of the indirect presentation pathway and chronic rejection in kidney, lung and hearts allografts.[10,24] We also conclude from our data that the direct presentation pathway does not predict GVD, while the indirect pathway appeared to correlate with GVD after HTx.

In the second year after HTx, the PBMC reactivity to indirectly presented TET antigens tends to normalize, possibly as a consequence of the reduction in immunosuppressive load given to the patient. To investigate these late PBMC response in more detail, we studied the PBMC reactivity to both presentation routes in a period of immunological quiescence (4 months to 2 years after HTx). The

response of 3 out of 5 patients (60%) to donor antigens presented by the direct pathway or TET antigens presented by the indirect pathway decreased compared to the day of transplantation. This high percentages of hyporesponsiveness was also described one year after human cadaver kidney transplantation,[25,26] while Reinsmoen et al.[27] demonstrated hyporeactivity in only 15% of cadaver kidney transplants and 36% of the living related transplants. This low percentage may be explained by the usage of homozygous typing cells as stimulator instead of donor cells in MLC. We think it would be reasonable to postulate that patients whose PBMC showed a hyporeactivity against both direct presented donor antigens and indirect presented TET antigens could have their immunosuppression reduced or even withdrawn. In patients with high responses it is probably unwise to taper their immunosuppressive schedule. Efforts should be undertaken in these patients to suppress these responses by changing their immunosuppressive protocol in attempt to prevent chronic rejection. Therefore, the assessment of presentation routes may be useful to individualize the maintenance of immunosuppressive therapy.
In conclusion, monitoring the direct and indirect presentation routes could be relevant tools to define the immunological status of transplant recipients.

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Chapter 5.2

Donor-specific helper T-lymphocyte and cytotoxic T-lymphocyte frequencies in peripheral blood in relation to graft vascular disease after clinical heart transplantation

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Based on:
NM van Besouw, CR Daane, LMB Vaessen, T van Gelder, B Mochtar, AHMM Balk, W Weimar. **Nonspecific immune reactivity of peripheral blood mononuclear cells is related to graft vascular disease.** *Transplant Proc 1997; 29: 2544-2545.*
NM van Besouw, CR Daane, P de Kuiper, T van Gelder, B Mochtar, AHMM Balk, LMB Vaessen, W Weimar. **Donor-specific CTL frequencies in peripheral blood in relation to graft vascular disease after clinical heart transplantation.** *Transpl Int 1998; 11 (1): S364-S366.*

Abstract

Cellular mechanisms may play a role in the development of graft vascular disease (GVD). We previously demonstrated that GVD correlates with parameters measured in graft-infiltrating lymphocytes.
In the present report, we investigated whether there is a correlation between the development of GVD and the frequency of donor-specific helper T-lymphocyte precursors (pHTL) and cytotoxic T-lymphocyte precursors (pCTL) in peripheral blood mononuclear cells (PBMC). We tested PBMC samples of 5 patients with GVD and 5 patients without GVD in the periods 3 to 6 months, 1 year and 3 years after heart transplantation (HTx). At all time points, GVD was not related to the number of pHTL and pCTL.
In conclusion, donor-specific cellular tests in peripheral blood could not be related to GVD. Apparently, donor-specific reactions associated with the induction of GVD can only be monitored in the graft.

Introduction

While the morphology of GVD (graft vascular disease) is well recognized, the mechanisms leading to GVD are still largely unknown. Both immunological and non-immunological factors may play a role in the development of GVD. We focused on donor-specific cellular test systems both intragraft and in peripheral blood in an attempt to find associations with GVD.

In a previous study, we demonstrated that increased T-helper 1 cytokine (IL-2 and IFN-γ) production by donor-stimulated graft-infiltrating lymphocyte cultures derived from endomyocardial biopsies taken within the first postoperative year is associated with GVD diagnosed at one year after heart transplantation (HTx).[1] These T-helper 1 cytokines give help to the differentiation and maturation of cytotoxic T-lymphocytes (CTL). In the infiltrating cells, GVD could also be related to the frequency of CTL.[2]

Because functional tests in the graft, necessitating culturing graft-infiltrating lymphocytes, are invasive and time-consuming, we performed experiments in peripheral blood. In heart transplant patients, high responses of increased numbers of helper T-lymphocytes (HTL) have been reported in relation to acute rejection.[3] The relationship between HTL in peripheral blood and GVD is unknown. Several studies have demonstrated that a reduction in circulating HTL and/or CTL is associated with stable engraftment after kidney, liver and heart transplantation.[4-6] Therefore, we wondered whether the frequency of HTL and CTL in PBMC (peripheral blood mononuclear cells) might also reflect an unstable situation as GVD. We stimulated the PBMC with donor cells to determine the total pool of CTL in peripheral blood. Because circulating HTL and CTL are predominantly precursors cells and not their mature committed cells,[7] we considered the measured number of HTL and CTL as HTL precursors (pHTL) and CTL precursors (pCTL) and related their frequency to the development GVD.

Materials and methods

Patients

We tested a total number of 15 PBMC samples from 5 patients with GVD and 5 patients without GVD in the period 3 to 6 months, 1 year and 3 years after HTx. All samples were taken at the day an endomyocardial biopsy was performed. To avoid bias of acute rejection, only those blood samples were analyzed that had no histological signs of myocytolysis in their accompanying biopsy.

All patients received standard immunosuppressive therapy, consisting of cyclosporin A (CsA) and low dose prednisone. One year after HTx, GVD was visually assessed on coronary angiograms by one of us (A.H.M.M.B.) and defined as all vascular wall changes including minimal wall irregularities.[8]

HTL- and CTL-assay

Responder PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Isopaque. PBMC were stimulated with phytohemagglutinin M (PHA; 1:100 final dilution; Difco Laboratories, Detroit, MI) to control the viability of the cells.

The frequency of IL-2 producing T-helper lymphocytes (HTL) was determined using the limiting dilution analysis assay described by Schanz et al.[9] with minor modifications.
Limiting dilution cultures were set up in 96-well U-bottom tissue culture plates (Costar, Cambridge, Mass.). 24 replicates of graded number PBMC responders were titrated in 7-step double dilutions starting from 2.5×10^4 to 391 PBMC/well and stimulated with an irradiated (30 Gy) and washed Epstein Barr transformed B-cell line (B-LCL) of donor origin (5×10^3 cells/well) in 200 µl culture medium (RPMI-1640 DM (Life Technology, Paisley, Scotland) supplemented with 10% pooled human heat-inactivated serum, 4 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin).
After 3 days of culture, 100 µl of the supernatant was harvested to test for IL-2 in a bio-assay using the IL-2-dependent CTLL-2 cell line as indicator system. HTL-frequency (HTLf) was determined as described by Strijbosch et al.[10]
The remaining cells were refreshed with 100 µl culture medium containing 40 U/ml recombinant IL-2 (final concentration 20 U/ml IL-2) (Biotest AG, Dreieich, Germany). After another 7 days of culture, we performed a cell mediated lympholysis. Each well was tested for cytolytic activity against 2.5×10^3 ^{51}Cr-labelled T-cell blasts of donor origin. These T-cell blasts can only be used as HLA class I target, and not as HLA class II target.[11] After 4 hours of incubation at 37 °C in 5% CO_2 the supernatants were harvested (Skatron harvesting system: Skatron-AS, Lierse, Norway) and the release of ^{51}Cr was determined in a γ-counter (Packard Instruments, Downers Grove, IL, USA). Maximum and spontaneous release were determined in 4-fold and defined by incubation of target cells with culture medium in the presence and absence of Triton X-100 (5% v/v 4-fold in TRIS buffer), respectively.
The percentage of lysis was calculated according to the formula:

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

The microcultures were considered cytolytic when the percentage lysis exceeded 10%.

Results

All PBMC samples reacted to donor cells in the HTL-assay. The pHTLf fluctuated per patient in time after HTx. At 3 to 6 months, 1 year and 3 years after HTx, no differences were found in the pHTLf of the PBMC between patients with and without GVD (Table 1).

Table 1 Frequencies of precursor helper T-lymphocytes (pHTLf) of peripheral blood mononuclear cells (PBMC) samples taken at different time points after heart transplantation (HTx) from patients with (n=5) and without (n=5) graft vascular disease (GVD).

	3-6 months after HTx	1 year after HTx	3 years after HTx
GVD-	128 (28-356)#	290 (56-337)	109 (16-981)
GVD+	106 (1-362)	57 (5-359)	206 (1-1105)

#Data are presented as the median pHTLf per 10^6 PBMC with the range in parentheses.

Donor-specific pCTL for HLA class I antigens were detected in PBMC of both patients with and without GVD. The number of pCTL fluctuated per patient in time after HTx, too. At 3 to 6 months, 1 year and 3 years after HTx, we also found no differences in the frequency of donor-specific pCTL (pCTLf) between PBMC derived from patients with GVD and patients without GVD (Table 2).
Analyzing the individual increase or decrease in pHTLf and pCTLf also did not result in differences between the patient groups.

Table 2 Frequencies of precursor cytotoxic T-lymphocytes (pCTLf) of PBMC samples taken at different time points after heart transplantation (HTx) from patients with (n=5) and without (n=5) graft vascular disease (GVD).

	3-6 months after HTx	1 year after HTx	3 years after HTx
GVD-	57 (11-302)#	142 (0-192)	31 (0-199)
GVD+	224 (0-1150)	197 (11-1442)*	126 (0-3079)

#Data are presented as the median pCTLf per 10^6 PBMC with the range in parentheses.
*one PBMC sample was inviable according to the PHA stimulation

Discussion

To elucidate whether a quantitative change of alloreactive pHTL and/or pCTL in PBMC is one of the mechanisms accounting for the development of GVD, we determined the frequency of pHTL and pCTL specific for donor HLA class I antigens of PBMC samples from patients with GVD and without GVD.
Several studies have demonstrated that a reduction in circulating HTL and/or CTL is associated with stable engraftment.[4-6] Interestingly, our study suggests that in patients with stable engraftment, who remained free from GVD, no consistent reduction in circulating pHTL or pCTL numbers was observed in time. We also found that in an unstable situation, such as GVD development, both the number of pHTL and pCTL and the variability in time of frequencies were not different from the results of the patients without GVD. Apparently, the presence of a pool of pHTL or pCTL specific for donor HLA class I antigens in PBMC does not reflect GVD development. So, it seems that parameters of an activated immune system in peripheral blood do not parallel the pathogenesis of GVD. What about the immune reactivity in the graft? We found that graft-infiltrating lymphocytes derived from patients with GVD produced more IL-2 and IFN-γ after donor-specific stimulation in the first year after HTx.[1] This increase of T-helper 1 cytokines in the graft from patients with GVD is not reflected in peripheral blood by an increment of IL-2 producing pHTL. We have investigated the

possible role of CTL in lymphocytes that infiltrate the transplanted heart. The frequency of *in vivo* primed or committed CTL with specificity for donor HLA class I in these graft-infiltrating cell cultures in our hands again did not discriminate between patients with and without GVD.[2] However, we found that a high frequency of CTL with specificity for donor HLA class II in the cultures may protect against the development of GVD.[2] We hypothesized that these particular CTL act by killing HLA class II bearing donor cells (endothelial cells and smooth muscle cells) responsible for GVD induction. In case of GVD development, the frequency of donor-directed HLA class II specific CTL is too low to lyse the donor cells. Activated donor endothelial cells secrete growth factors which promote donor smooth muscle cell proliferation leading to intima thickening. In other words, we think that GVD is an auto-reactive phenomenon of the donor tissue induced by acceptor derived cytokines. We have not yet measured the number of pCTL specific for donor HLA class II antigens in PBMC. Further studies on this hypothesis of donor auto-reactivity could be important for understanding the pathogenesis of GVD.
In summary, the frequency of circulating donor-specific pHTL and pCTL with donor HLA class I specificity does not correlate with the development of GVD. Only donor-specific T-helper 1 production and the frequency of CTL specific for donor HLA class II antigens in the graft are associated with the induction of GVD.

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Chapter 6

Summary and conclusions

The efficacy of heart transplantation for end-stage cardiac disease has generally been accepted. The patient survival rates after the 1st, 5th and 7th year are in the region of 92%, 84% and 77%, respectively.[1] However, despite these good results, many problems remain unsolved, e.g. the exact etiology of acute and chronic rejection processes.

In *chapter 1* a general introduction is given to possible cellular processes leading to the immune response directed to the allograft. Not only the difference of HLA antigens between allograft recipient and donor are important during rejection, but also the presentation pathway of donor HLA to recipient cells. Therefore, the direct and indirect antigen presentation pathways are described *(chapter 1.4)* and investigated *(chapter 5.1)*. Cytokines and cytotoxic T-cells (CTLs) are also core topics in this thesis. Their role is discussed in the introduction in relation to clinical transplantation. In *chapter 2* a brief overview of literature in relation to peripheral blood monitoring after transplantation and to monitoring of graft-infiltrating lymphocytes (GIL) propagated after clinical heart transplantation is provided.
The major theme of the studies described in this thesis is: intragraft *(chapter 3 and 4)* and peripheral blood monitoring *(chapter 5)* in relation to both acute rejection *(chapter 3 and 5)* and graft vascular disease (GVD) *(chapter 4 and 5)*.

In the first part of *chapter 3*, it describes how GIL-cultures can be propagated from endomyocardial biopsies (EMBs) independent of acute rejection. It is also shown that no difference in phenotypic composition was found between acute rejection and non-rejection EMBs *(chapter 3.1)*. Additionally, this chapter shows that cell growth is significantly more often obtained from EMBs derived cell cultures with signs of acute rejection than those without rejection. In these cell cultures $CD4^+$ cells were always more numerous than $CD8^+$ cells.
In *chapter 3.2* a clear correlation between mRNA expression and the production of cytokines is demonstrated in the GIL-cultures. In *chapter 3.3* the exact kinetic of one T-helper 1 (IL-2) and one T-helper 2 (IL-4) cytokine is described. In this chapter, it is further shown that each individual GIL-culture has its own characteristic mRNA and protein profile. Stimulating the cultures for 20 hours with donor-antigens was a prime method to compare cytokine production patterns between different groups of cultures, e.g. cultures derived from acute rejection EMB versus non-rejection EMB and those cultures from patients who developed GVD versus who did not. Because significantly more cytokines were produced after donor stimulation than after third-party stimulation, a donor-specific cytokine pattern can be determined *(chapter 3.2, 3.3 and 3.4)*.
Cytokines play an important role in acute rejection. Therefore, the production of cytokines by cells infiltrating the allograft (GIL) was investigated during acute

rejection and in a rejection-free period *(chapter 3.4)*. In the first 3 postoperative months significantly higher levels of T-helper 1 (IL-2 and IFN-γ) cytokines were produced after donor-specific stimulation of GIL-cultures derived from rejection biopsies compared to those from non-rejection biopsies. These T-helper 1 cytokines enhance the generation of CTLs,[2] which can recognize and kill donor cells leading to histologic signs of acute rejection. In addition, the high levels of IFN-γ produced by the GIL can upregulate HLA class II expression.[3] During an acute rejection episode HLA class II expression is increased,[4] probably induced by IFN-γ produced by GIL, resulting in an infiltration of the graft with lymphocytes.
After this period, the cytokine levels produced by the GIL were comparable. Interestingly, the high T-helper 1 cytokine levels produced in this later period after transplantation were in the range of the high levels of GIL taken from rejection biopsies in the first 3 postoperative months. Probably a higher dose of immunosuppression is necessary to prevent rejection in the first 3 months after transplantation than at any later stage, resulting in a suppressive effect on the T-helper 1 cytokines produced by GIL. With regard to the T-helper 2 cytokines, IL-4 and IL-6, no relation was found to the presence of acute rejection at any time after transplantation. Accordingly, these results demonstrated that in the first 3 months after transplantation, a high production of T-helper 1, and not T-helper 2 cytokines correlated with acute rejection. Thereafter, a continuous state of immunological activity is seen even in the absence of acute rejection, resulting in high T-helper 1 cytokine production of the GIL.

One year after transplantation, 26% of the patients had chronic rejection diagnosed in the coronary arteries by angiography. This finding increased proportionally to almost 80% 10 years after transplantation.[1] Chronic rejection is also called accelerated coronary atherosclerosis or graft vascular disease (GVD).
Acute rejection cannot be associated with the development of GVD *(chapter 4.1)*. Also no correlation was found between GVD and either GIL growth or the phenotypic composition of the GIL-cultures *(chapter 4.1)*.
Cytokines produced after stimulating the GIL-cultures with donor cells were investigated in relation to the development of GVD *(chapter 4.2)*. A clear correlation was found between early cytokine production and GVD. Significantly higher levels of T-helper 1 cytokines (IL-2 and IFN-γ) were produced by GIL-cultures from patients who developed GVD in the first year after transplantation, but there was no higher production of the T-helper 2 cytokines IL-4 and IL-6. Early IL-2 production stimulates the activation and proliferation of lymphocytes.[5] IFN-γ recruits macrophages[6] into the graft and enhances CTL differentiation[6-8] along with the MHC expression of the vascular endothelium.[3] This endothelial cell activation by the T-helper 1 cytokines will finally lead to the characteristic manifestation of GVD: intima thickening.
More than one year after transplantation, the T-helper 1 cytokine production no

longer discriminates between patients with and without GVD. These results emphasize the importance of measuring cytokine production levels during the first year after transplantation, which give an earlier warning of GVD before the angiogram routinely performed on the first anniversary of the transplant. However, after this first postoperative year, detectable IL-6 levels were produced more often by the GIL-cultures of patients with signs of GVD in their angiogram versus the cultures from patients who had no signs of GVD after one year. The IL-6 produced by the GIL probably continues the process of intima thickening in the vessels of the donor heart. Because IL-6 promotes the proliferation of smooth muscle cells,[9] this may be a co-factor in the accelerated process of coronairsclerosis. Consequently, GVD is an immune-mediated response to donor antigens and the development of GVD is initiated by T-helper 1 cytokines produced by GIL early after transplantation, while the IL-6 production of GIL maintains and accelerates GVD.

Because T-helper 1 cytokines enhance CTL differentiation,[6-8] CTLs propagated from EMBs could also play a role in GVD development. The frequency of GIL-cultures lytic to donor HLA class I and class II antigens did not discriminate between the patients with and without GVD in the first year after transplantation *(chapter 4.3)*. In addition, the frequency of *in vivo* primed or committed CTLs (cCTLs) specific for donor HLA class I antigens present within the graft did not differentiate between the patients with GVD and those without. The cultures contained predominantly cCTLs with low avidity for donor HLA class I antigens, which was also irrespective of the development of GVD one year after transplantation. However, in patients who did not develop GVD in the first postoperative year, the frequency of cCTLs with donor HLA class II specificity was significantly higher than in those patients who did. Apparently, a high frequency of donor HLA class II-directed cCTLs protects for GVD.
As a possible explanation for this phenomenon, the following model was suggested. Nonspecific tissue damage shortly after transplantation results in macrophage infiltration and cytokine release followed by upregulation of HLA class I and class II and lymphocyte binding ligands on donor cells.[10-12] Subsequently, donor-specific activated cells (GIL) enter the graft. GIL derived from patients with GVD in the first postoperative year produce high levels of T-helper 1 cytokines, resulting in an upregulation of HLA class II antigens on vascular wall cells. HLA class II directed cCTLs accumulate in the transplant at the site of the activated donor cells on the vascular wall. Thereafter, donor cells with HLA class II antigen on their surface, which are responsible for GVD (endothelial cells and smooth muscle cells), are killed and GVD is prevented. In case the donor-directed HLA class II cCTL frequency is too low to lyse the activated vascular wall cells, GVD will develop.

Because the EMB-procedure is an invasive method and propagating GIL-cultures is time-consuming, relations with peripheral blood monitoring and acute rejection and

the development of GVD are investigated *(chapter 5)*.
In *chapter 5.1* the relevance of measuring the direct and indirect presentation pathway was studied. The direct pathway was determined by the proliferative reactivity of peripheral blood mononuclear cells (PBMC) to donor cells. Complete removal of donor antigen-presenting cells from the stimulator population in a mixed lymphocyte culture is not a suitable tool for measuring indirect presentation of alloantigens. In contrast, this latter route could easily be activated by the nominal antigen tetanus toxoid. Analysing the level of PBMC reactivity showed that both the immune response via the direct and indirect presentation pathway increased, when cyclosporin A levels inadvertently decreased to inadequate concentrations and acute rejection was diagnosed.
The direct presentation route does not predict GVD, while the indirect pathway appeared to correlate with GVD after transplantation.
During immunological quiescence high and low responders were detected to both pathways. Therefore, the assessment of presentation routes in peripheral blood may be used as a tool for selecting patients in which the tapering of immunosuppression can safely be applied.

In the graft T-helper 1 cytokines and CTLs play a role in the development of GVD *(chapter 4.2 and 4.3)*. In *chapter 5.2* the frequency of donor-specific helper T-lymphocytes (HTLs) and CTLs in peripheral blood were related to GVD. Both the presence of a pool of HTLs and CTLs specific for donor HLA class I antigens did not reflect GVD development. Currently, the role of CTL specific for donor HLA class II antigens in peripheral blood remains illusive.

In conclusion, during acute rejection the response via directly presented donor antigens and indirectly presented tetanus toxoid antigens increased in peripheral blood. This latter presentation route is also associated with GVD. Donor-specific cellular tests such as the frequency of HTLs and CTLs could not be related to GVD in peripheral blood. In the graft, high levels of donor-specific T-helper 1 cytokine production not only correlated with acute rejection, but also with the development of GVD. The onset of GVD is prevented by high numbers of CTL specific for donor HLA class II antigens.

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Chapter 7

Samenvatting

7.1. Samenvatting

In dit proefschrift zijn T-cel functies na klinische harttransplantatie beschreven. Aandacht is vooral besteed aan verschillen in T-cel functies tussen episodes met en zonder acute afstotingsverschijnselen en verschillen tussen patiënten die wel of geen versnelde coronairsclerose (VCS) ontwikkelen. VCS is een manifestatie van chronische afstoting in het getransplanteerde hart.
Beide afstotingsprocessen zijn gerelateerd aan eigenschappen van zowel transplantaat-infiltrerende lymfocyten (TIL) kweken als van perifeer bloed mononucleaire cellen (PBMC). Van de TIL-kweken werd het groeipatroon, het fenotype, de cytokinenproduktie en de cytotoxische capaciteit geanalyseerd. In de PBMC werden de direkte en indirekte alloantigeen-presentatie routes gemeten, en de hoeveelheid T-helper cellen en cytotoxische T-cellen bepaald.

Acute afstoting in het transplantaat

TIL kunnen zowel verkregen worden uit afstotingsbiopten als niet-afstotingsbiopten. Groei is echter vaker waargenomen in kweken die afkomstig zijn van afstotingsbiopten *(hoofdstuk 3.1)*. Het fenotype van de TIL is onafhankelijk van afstoting *(hoofdstuk 3.1)*.
In de TIL-kweken is wat cytokinen betreft op gen- en eiwitniveau gedifferentieerd tussen donor en derde-partij reactiviteit *(hoofdstuk 3.2 en 3.3)*. De gemeten cytokinenpatronen in de kweken zijn een afspiegeling van donor-specifieke processen in het getransplanteerde hart en niet van de algemene immuunreactiviteit. Na stimulatie met donorcellen, worden in de eerste 3 maanden na transplantatie meer T-helper 1 (IL-2 en IFN-γ) cytokinen gemaakt door TIL-kweken afkomstig van afstotingsbiopten dan door de kweken van biopten zonder afstotingsverschijnselen *(hoofdstuk 3.4)*. Vanaf 3 maanden is de produktie van de T-helper 1 cytokinen in de kweken van niet-afstotingsbiopten even hoog als in de kweken van de afstotingsbiopten. In deze periode is de produktie zelfs vergelijkbaar met de hoge produktie van de TIL-kweken afkomstig van de afstotingsbiopten afgenomen in de eerste 3 maanden na transplantatie. Voor de T-helper 2 cytokinen IL-4 en IL-6 is geen relatie met acute afstoting waargenomen. Deze resultaten suggereren dat T-helper 1, en niet T-helper 2 cytokinen, in de eerste 3 maanden na de operatie geassocieerd zijn met acute afstoting. Hierna treedt een staat van continue immunologische activiteit op, zelfs in de afwezigheid van afstoting.

Versnelde coronairsclerose in het transplantaat

Geen verschil is aantoonbaar tussen patiënten met en zonder VCS voor wat betreft de groei van lymfocyten uit endomyocardbiopten *(hoofdstuk 4.1)*. Ongeacht de herkomst van de biopten worden er altijd meer $CD4^+$ dan $CD8^+$ T-cellen in de

kweken waargenomen. Het fenotype tussen de verschillende patiëntengroepen verschilt niet wat betreft het percentage cellen dat de T-cel receptor-αβ en -γδ, CD3, CD4, CD8 of CD16 op hun oppervlak draagt *(hoofdstuk 4.1)*.

Niet alleen werd een relatie tussen acute afstoting en cytokinenpatronen gevonden *(hoofdstuk 3.4)*, maar ook een verband tussen deze patronen en de ontwikkeling van VCS *(hoofdstuk 4.2)*. Na donor-specifieke stimulatie van TIL-kweken, worden in het eerste jaar na transplantatie significant meer T-helper 1 (IL-2 en IFN-γ), maar geen T-helper 2 (IL-4 en IL-6) cytokinen, gevonden bij patiënten die tekenen van VCS vertonen in het één-jaars angiogram dan bij patiënten die hiervan vrij blijven. Langer dan één jaar na transplantatie, wordt deze relatie met T-helper 1 cytokinen niet meer gevonden, maar wordt significant meer van het T-helper 2 cytokine IL-6 gemaakt door de kweken afkomstig van patiënten met VCS. Hieruit is te concluderen dat de verhoogde, maar voorbijgaande, T-helper 1 cytokinen produktie de aanzet kan zijn voor het ontstaan van VCS (activatie van endotheelcellen). Nadat de zichtbare tekenen van VCS in het één-jaars angiogram zijn ontstaan, kan IL-6, een mitogeen voor gladde spiercellen, het proces van gladde spiercelproliferatie in stand houden of verder versnellen.

Omdat T-helper 1 cytokinen de activatie van cytotoxische T-cellen (CTLs) bevorderen, zijn in de TIL-kweken de frequentie en aviditeit van reeds *in vivo* geactiveerde CTLs (cCTLs) gericht tegen donor klasse I en II HLA antigenen bepaald en gerelateerd aan de ontwikkeling van VCS *(hoofdstuk 4.3)*. In het eerste jaar na transplantatie kon de frequentie van cCTLs gericht tegen HLA klasse I antigenen in de kweken niet differentiëren tussen patiënten met en zonder VCS. De frequentie van cCTLs gericht tegen donor klasse II antigenen was echter hoger in de kweken afkomstig van patiënten die één jaar na transplantatie gave coronairvaten vertoonden. Bij de reactiviteit tegen klasse I antigenen overheersten in de kweken van beide patiëntengroepen de cCTLs met een lage aviditeit. Uit deze resultaten wordt geconcludeerd dat een hoge frequentie van cCTLs die gericht zijn tegen donor HLA klasse II antigenen de ontwikkeling van VCS tegengaan. Mogelijk zijn deze cellen cytotoxisch voor geactiveerde donor cellen, zoals endotheel en gladde spiercellen, die VCS mede kunnen veroorzaken.

Acute afstoting en versnelde coronairsclerose in het perifeer bloed

Zowel de direkte en indirekte route van alloantigeen-presentatie kunnen van belang zijn bij acute afstoting en VCS *(hoofdstuk 5.1)*. De direkte presentatie route is gemeten door ontvanger PBMC in een MLC ('mixed lymphocyte culture') met donorcellen te stimuleren. Indien er geen donor antigeen-presenterende cellen (APC) in een MLC aanwezig zijn, kan geen stimulatie via de direkte presentatie route plaatsvinden en moet de reactie dus via ontvanger APC verlopen (indirekte route). De indirekte route was niet detecteerbaar na stimulatie met donorcellen waaruit de APC verwijderd zijn gemeten worden, maar werd bepaald door PBMC te stimuleren

met het nominaal antigeen tetanus toxoïde (TET). PBMC van patiënten vóór transplantatie kunnen zowel op antigeen gepresenteerd via de direkte als via de indirekte (TET) route reageren. Na transplantatie zijn nog maar weinig PBMC monsters hiertoe in staat en deze reageren dan voornamelijk op direkt gepresenteerde donor antigenen. Immunosuppressie beïnvloedt dus de presentatie routes. Dit blijkt ook uit de hoogte van de reactiviteit op een tijdstip dat te lage, ineffectieve concentraties cyclosporine A in het plasma worden gevonden, waardoor acute afstoting optreedt. Op dat moment stijgt de PBMC reactiviteit na stimulatie met direkt gepresenteerde antigenen en wordt ook de indirekte route meetbaar.
PBMC monsters afkomstig van patiënten met VCS reageerden sterker tegen het indirekt gepresenteerde TET-antigeen, en niet tegen direkt gepresenteerde donorantigenen, dan PBMC monsters van patiënten die geen VCS ontwikkelden waargenomen in het één-jaars angiogram.
In een immunologische rustige periode reageerden sommige patiënten heftig en andere patiënten verminderd tegen direkt en indirekt gepresenteerde antigenen. Het monitoren van deze routes kan dan ook van belang zijn bij het bepalen van de immunologische status van de patiënt.

In het transplantaat spelen T-helper 1 cytokinen en CTLs een rol bij het ontstaan van VCS *(hoofdstuk 4.2 en 4.3)*. Daarom werd in *hoofdstuk 5.2* onderzocht of in perifere bloedcellen eveneens een relatie met de helper T-lymphocyten (HTLs) en/of CTLs en VCS aantoonbaar is. Vóór, noch na diagnose van VCS zijn in de frequentie van HTLs en CTLs verschillen te vinden tussen PBMC van patiënten met en zonder tekenen van VCS in het één-jaars angiogram.
Hieruit blijkt dat donor-specifieke immuunprocessen in relatie tot VCS alleen in het transplantaat (TIL-kweken) te meten zijn.

7.2. Populaire samenvatting

Harttransplantatie is voor veel patiënten in het eindstadium van hartlijden de enig mogelijke behandelvorm. Na transplantatie zijn zowel acute als chronische vormen van afstoting een groot probleem. Vroeg na transplantatie kan acute afstoting van het getransplanteerde hart voorkomen worden met medicijnen die het afweersysteem onderdrukken. Later na transplantatie kunnen, als gevolg van chronische afstoting, vaatafwijkingen in de kransslagaderen van het getransplanteerde hart (versnelde coronairsclerose: VCS) ontstaan, hetgeen op den duur een belangrijke doodsoorzaak is bij de transplantatie patiënten. In het Academisch Ziekenhuis Rotterdam-Dijkzigt treedt bij 75% van de patiënten een periode van acute afstoting op en vertoont 26% van de patiënten één jaar na transplantatie tekenen van VCS.
Acute afstoting en waarschijnlijk ook de afwijkingen in de vaten zijn het resultaat van cellulaire reacties. In Rotterdam worden regelmatig stukjes hartweefsel (biopten) na transplantatie afgenomen. Uit deze biopten kunnen infiltrerende cellen, afkomstig van de transplantaat-ontvanger, gekweekt worden. Deze cellen zijn verantwoordelijk voor de afstotingsprocessen. Ook met behulp van cellen die geïsoleerd zijn uit het bloed van de transplantatie patiënten kunnen afstotingsprocessen bestudeerd worden.

De vraagstellingen die in dit proefschrift onderzocht werden zijn:

- Welke zichtbare verschillen *(hoofdstuk 3.1)* zijn er tussen de kweken van biopten met en zonder tekenen van acute afstoting en is het vermogen tot uitscheiden van regulerende stoffen (cytokinen) door de gekweekte cellen verschillend *(hoofdstuk 3.2, 3.3 en 3.4)*?
- Bevatten kweken van biopten verkregen van patiënten met en zonder VCS andere celtypen *(hoofdstuk 4.1)*, scheiden de kweken verschillende cytokinen uit *(hoofdstuk 4.2)* en is hun vermogen om donorcellen te doden verschillend *(hoofdstuk 4.3)*?
- Verschillen de patiëntengroepen (wel/geen acute afstoting, wel/geen VCS) in de manier waarop donor-eiwitten aan de cellen in het bloed van de transplantaat-ontvanger worden gepresenteerd *(hoofdstuk 5.1)*? Is in het bloed de hoeveelheid cellen die tegen donorcellen gericht zijn verschillend tussen patiënten met en zonder VCS *(hoofdstuk 5.2)*?

Dit proefschrift maakt duidelijk dat celgroei vaker optreedt uit biopten met afstotingsverschijnselen dan uit biopten zonder afstoting. Het al dan niet hebben van VCS heeft hier geen invloed op. Het celtype in de kweken is tussen de verschillende patiëntengroepen vergelijkbaar.

De cytokinen gemaakt door de celkweken van de biopten laten een relatie met acute afstoting én met het ontstaan van VCS zien.
Cellen van afstotingsbiopten maken meer cytokinen (IL-2 en IFN-γ) dan cellen afkomstig van biopten zonder tekenen van afstoting. Deze cytokinen zorgen onder andere voor de aanmaak van cellen die in staat zijn donorweefsel te doden.
Cellen van patiënten die één jaar na transplantatie tekenen van VCS vertonen produceren gedurende dat eerste jaar ook meer cytokinen (IL-2 en IFN-γ) dan de patiënten zonder VCS. Deze cytokinen kunnen eveneens cellen in de vaatwand van de kransslagaderen van het getransplanteerde hart beschadigen: het eerste kenmerk van VCS. Een ander cytokine, IL-6, wordt later dan één jaar na transplantatie in hogere frequentie gemaakt door de kweken van patiënten die zichtbare coronaire vaatafwijkingen vertonen. Omdat onder invloed van het cytokine IL-6 spiercellen in de vaatwand van de kransslagaderen kunnen vermenigvuldigen, kan IL-6 het proces van VCS in stand houden en mogelijk versnellen.
De gekweekte cellen verschillen ook in hun celdodende activiteit. In de celkweken van patiënten zonder VCS bleken meer ontvangercellen gericht tegen donorcellen te zitten dan bij de kweken van patiënten met VCS. De donorcellen in de vaatwand die betrokken zijn bij het ontstaan van VCS worden waarschijnlijk door deze cellen opgeruimd, zodat deze patiënten vrij van VCS blijven.

Indien de medicijnen, die het afweersysteem onderdrukken, per ongeluk te lage waarden in het bloed krijgen en acute afstoting wordt waargenomen worden donor-eiwitten en ook andere eiwitten, zoals tetanus-eiwitten, makkelijker gepresenteerd aan bloedcellen van de transplantaat-ontvanger dan wanneer er geen afstoting is.
Bij cellen uit het bloed van patiënten met VCS wordt in vergelijking met de cellen van patiënten zonder VCS alleen een verschil gemeten in de presentatie van tetanus-eiwitten, de presentatie van donor-eiwitten is vergelijkbaar.

In het bloed van de getransplanteerde patiënten is eveneens onderzocht of dezelfde parameters, als in de celkweken van de biopten, gerelateerd zijn aan het ontstaan van VCS. De resultaten van deze testen zijn in het bloed echter niet diagnostisch voor het optreden van VCS.
Hieruit is te concluderen dat processen specifiek gericht tegen donorweefsel met betrekking tot VCS alleen in het transplantaat (in de celkweken van biopten) voorkomen.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 29 maart 1968 te Zwijndrecht. In 1987 behaalde zij haar VWO-B diploma aan het Mencia de Mendoza Lyceum te Breda. Aansluitend begon zij met de studie Gezondheidswetenschappen (richting: Biologische Gezondheidskunde) aan de Rijksuniversiteit Limburg. Haar afstudeerstage op de Afdeling Klinische Immunologie van de Katholieke Universiteit Leuven (België) werd besteed aan 'De technische diagnosestelling van voedingsmiddelenovergevoeligheid' onder leiding van dr E. Stevens. In augustus 1991 behaalde zij het doctoraal examen. Vervolgens heeft zij een aanvullende doctoraalfase Gezondheidswetenschappen gevolgd, waarin zij twee onderzoeksstages liep bij de Afdeling Chronische en Infectie Ziekten van het Instituut voor Toegepaste Radiobiologie en Immunologie (ITRI)-TNO. Bij de eerste stage van september 1991 tot mei 1992 participeerde zij in onderzoek naar de immunoregulatie van autoimmuun-artritis onder leiding van dr N.P.M. Bakker en dr P.H. van der Meide. Tijdens de tweede stage (mei-november 1992) bepaalde zij onder leiding van dr R. Bontrop nucleotide sequenties van het HLA klasse II locus DM in verschillende niet-humane primaten.
In december 1992 trad zij in dienst van de Erasmus Universiteit Rotterdam bij de Afdeling Interne Geneeskunde I van het Academisch Ziekenhuis Rotterdam-Dijkzigt op het door de Nederlandse Hartstichting gefinancierde projekt 'Pathogenese van versnelde coronairsclerose (chronische afstoting) na harttransplantatie'. Op de resultaten van dit projekt is, onder leiding van Prof.dr W. Weimar, dit proefschrift gebaseerd. Vanaf september 1996 werkt zij in dienst van het Academisch Ziekenhuis Rotterdam-Dijkzigt op deze afdeling (o.l.v. Prof.dr W. Weimar) aan het door de Nierstichting Nederland gesubsidieerde projekt 'Donor-specifieke T-cel reactiviteit als leidraad voor verantwoorde vermindering van immunosuppressieve druk'.

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